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The *Journal of Pharmaceutical Sciences* considers manuscripts for publication in the following categories:

Review Articles—Summaries covering the more recent advances in an area in which the author has been actively engaged will be published as review articles. Such papers are intended to be critical evaluations of published reports which have previously appeared in the scientific literature.

Research Articles—Reports of original laboratory research in pharmacy will be considered for publication in this section. Pharmacy is defined here in the broadest sense, and includes pharmaceutically related investigations in allied basic disciplines.

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Communications—Short communications which report new findings of outstanding importance will be given prompt publication after review and acceptance. Corrections and reinterpretations of previously published reports will also be considered. Communications should generally be written in paragraph style, without figures, tables, or headings other than a title.

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The Editor comments

WALTER H. HARTUNG, Ph.D.

During his long career and many productive years, Dr. Walter H. Hartung was a most unusual individual in several respects. He was an accomplished educator, author, research scientist, and perhaps above all else, a fine gentleman.

A pioneer in the field of drug chemistry, one of several textbooks which he co-authored on the subject is said to be the first American book in this field. He enriched the faculties of four large universities through his services. In addition to his lectures he inspired and guided the graduate research of a large number of devoted students. The numerous high quality research publications, his various oral reports at scientific meetings, and the two volumes which he edited in the series titled "Medicinal Chemistry," all attest to the high degree of his competency in the field. This was recognized when the American Pharmaceutical Association conferred its highest research award, the Ebert Prize, upon him in 1947. The high offices which he held in the Division of Medicinal Chemistry of the American Chemical Society likewise indicate the great esteem in which he was held by his associates in the sister field of chemistry. He was recently elected to membership on the Committee of Revision of the *United States Pharmacopeia*, and has been serving as a member of the newly-formed A.M.A.-U.S.P. drug nomenclature committee.

Dr. Hartung was unique, however, in that he never sought honor, prestige, nor glory. On the contrary, his characteristic personal humility and gentle kindness made a deep impression on all with whom he came in contact.

We are deeply honored to carry as our review article in this issue, a paper titled *Stereochemistry*, which was probably the last manuscript he authored.

We sadly note the passing on September 29, 1961, of Walter H. Hartung, Ph.D.

Edward G. Feldmann

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Form of Presentation.—Titles of manuscripts should be kept as short as possible and preferably restricted to one line. The name of the laboratory in which the work described was done should be placed in a footnote at the end of the first page. Each manuscript should begin with one or more introductory paragraphs indicating the intention and meaning of the investigation and the method of procedure. The introduction should be followed by a report of the essential details of the experimental work, and a discussion or interpretation where necessary. Authors may find it helpful to arrange manuscripts to conform to the style of presentation as illustrated in papers on analogous subjects published in recent issues of THIS JOURNAL.

In preparing manuscripts for publication in THIS JOURNAL, center headings should be avoided as far as possible. Side headings followed by a period and dash should be indented and run into the part of the text to which they apply. Such side headings may be used as freely as desired. Literature citations should be numbered consecutively in order of appearance in the manuscript and should be indicated by full-sized Arabic numerals enclosed in parentheses. If a literature reference is cited more than once in a manuscript, subsequent citations should be indicated by the first number assigned. References to footnotes throughout the text should be numbered consecutively by superscript Arabic numerals, but such references in each table should be designated by superscript lower case letters beginning with "a" and continuing in natural order. Literature citations should be grouped at the end of the manuscript under the heading "References." All bibliographic references should be checked with the original publications. The citations should be numbered consecutively with full-sized Arabic numerals enclosed in parentheses corresponding to the numbers used in the text. The names of all periodicals cited in the list of references must be abbreviated in accordance with abbreviations given by **Chemical Abstracts** in its "List of Periodicals Abstracted." The sequence followed in the citations must be as follows:

(1) Doe, J. B., *Am. J. Physiol.*, **79**, 289(1927).

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Figures.—All drawings should be made in India ink, preferably on white tracing paper or cloth. Original drawings are preferable, but clear, well-prepared glossy photographic prints are acceptable. The size of illustrations should not exceed the 8.5″ x 11″ manuscript size. They should be designed for reduction to the width of one column (2.75″) or full page width (5.5″). Thus, height rather than width should be accentuated where possible.

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Tables.—Tables should be carefully constructed so that the data presented may be understood easily. The indiscriminate use of tables should be avoided. Tables should be constructed so as to occupy fully a single column (2.75″) which will take about 40–50 characters and spaces. Tables which require two columns are also acceptable. Interpretative statements should not be written on the body of a table but should be explained in footnotes. Column heads should be made as small as possible and standard abbreviations should be used liberally. Data which may easily be deduced by simple arithmetic from data in another column should not be included. All tables should be referred to in the text of the manuscript. Every table and every column should be supplied with an appropriate heading. It is not necessary to place tables on separate sheets, and in fact it is preferable to intersperse them in the manuscript. Tables should be numbered consecutively using Roman numerals. The table number and the title should be placed in a continuous heading above the data presented. The preparation of a clear and concise table of data requires much planning and infinite care.

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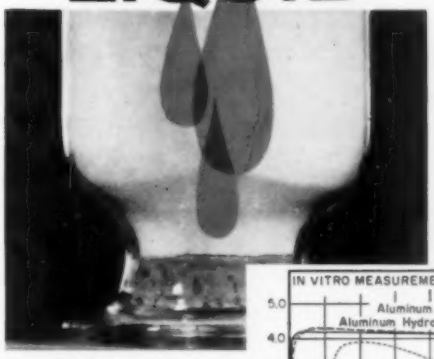

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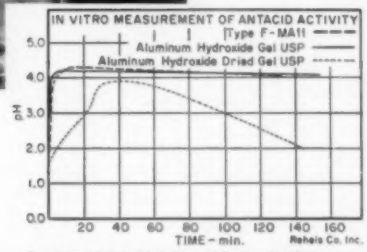

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NOTICE

PAPERS FOR SECTION MEETINGS OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

The next annual meeting of the American Pharmaceutical Association will be held in Las Vegas, Nevada, March 25–30, 1962. Titles and abstracts (not to exceed 200 words) of papers must be submitted to the appropriate Section secretary not later than January 1, 1962, or preferably earlier, in order to assure a place on any Section program. Papers should be sent to the appropriate Section secretary; the names and addresses of these secretaries are listed below.

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PRELIMINARY ANNOUNCEMENT OF THE SCIENTIFIC SECTION PROGRAM FOR THE 1962 A.Ph.A. MEETING

In addition to the usual general papers, four special sessions have been planned as part of the forthcoming Scientific Section Meeting at Las Vegas during the week of March 25, 1962. These symposia have been designed to offer maximum opportunity and time for stimulating discussions and controversy in limited pharmaceutical areas. The papers offered in these sessions will be preprinted and distributed to the Section membership prior to the meeting.

The four symposia and their respective chairmen are:

CHAIRMAN	TOPIC
J. V. Swintosky[1]	Absorption, Excretion, and Metabolism of Drugs
T. Higuchi[2]	Problems Related to Increasing Drug Solubility and Other Equilibria of Pharmaceutical Interest
E. R. Garrett[3]	Chemical Kinetics and Mechanism of Degradation of Drugs and Compounds of Biochemical Interest
S. M. Kupchan[4]	Biologically Active Natural Products and Derived Synthetic Drugs

If you have material you feel is suitable for any of these symposia, please write a short but informative summary of your work and send it to the appropriate chairman. This should be done by November 15, 1961. The presiding officers are also planning to solicit authoritative works.

Regular contributed papers will be accepted and presented as usual. The deadline for title and abstracts for these is January 1, 1962, and they should be sent directly to Dr. Anderson, the Section Secretary. Since we expect a full and worthwhile program, all contributions should be submitted as early as possible.

[1] Smith Kline & French Laboratories, Philadelphia 1, Pa.
[2] School of Pharmacy, University of Wisconsin, Madison 6.
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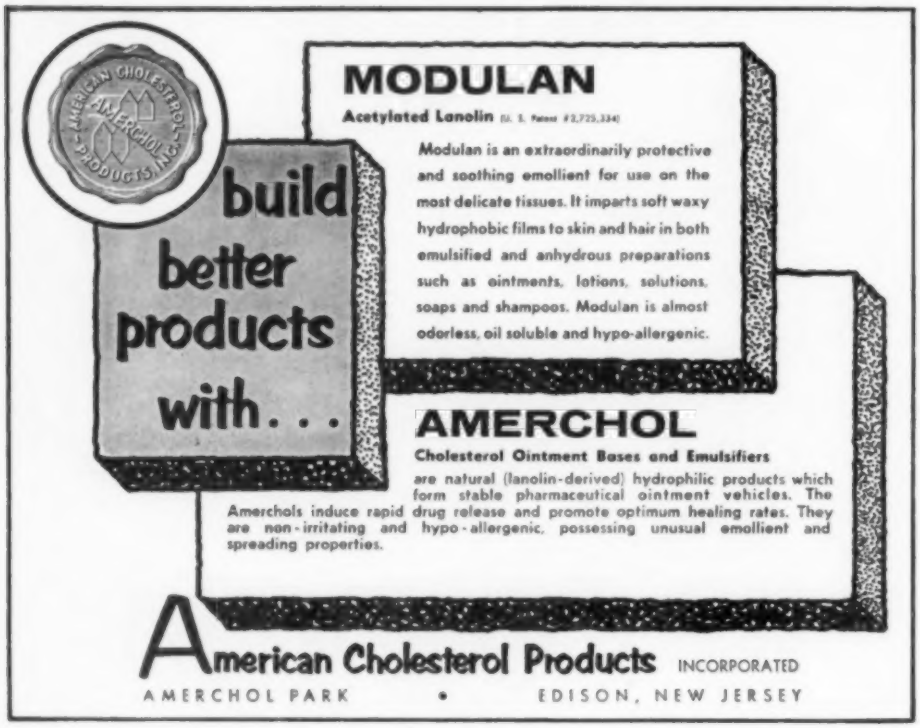
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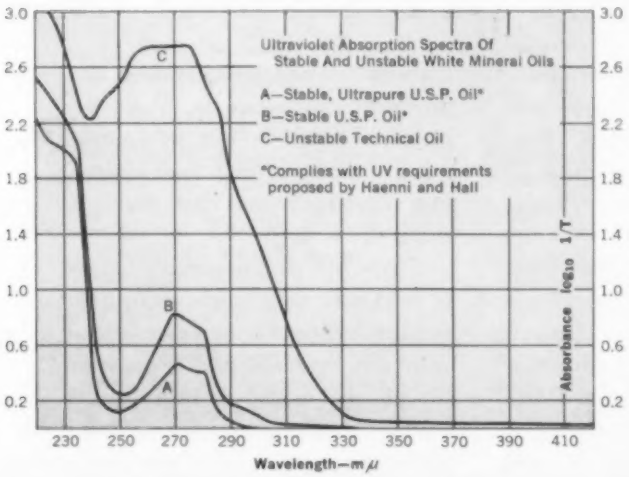

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JOURNAL OF Pharmaceutical Sciences

October 1961 volume 50, number 10

Review Article

Stereochemistry

By WALTER H. HARTUNG† and JOHN ANDRAKO

"Pursuit of the details of molecular structure and environment is the occupation of all chemists part of the time and part of the chemists all of the time."[1]

When Jean Baptiste Biot (1774–1862) observed in 1815 that substances such as sugar, tartaric acid, and oil of turpentine are capable of rotating a plane of polarized light, whether in the solid or liquid state, or in solution, or even (for turpentine) as vapor, he concluded that this revealed a hitherto unknown property inherent within molecules themselves.[2]

A further lustrous moment came to its climax when Louis Pasteur, under the skeptical eyes of the now venerable Biot, was obliged to separate from solid sodium ammonium tartrate the crystals with *dextro-* and *levo*-hemihedral facets; Biot himself eagerly examined first the *levo*-crystals for their effect on polarized light and thus observed, for the first time, as Pasteur had previously seen and now predicted, their *levo*-rotation.[3] Pasteur perceived that in the two different tartaric acids the "arrangements" are identical except that they exhibit symmetry in opposite directions and that he was dealing with arrangements that are not superposable.[4]

During the last third of the nineteenth century, the notion that molecules are three-dimensional permeated the atmosphere,[5] but the concept of how this might apply to organic molecules did not come into focus until 1874, at which time van't Hoff and LeBel, independently and almost simultaneously, suggested that the carbon atom itself be looked upon as tridimensional, with its four valences radiating, as it were, at equal angles from a common point of origin. Considerable ridicule greeted this hypothesis, but not for long; it soon provided a reasonable explanation for many otherwise puzzling observations.[6]

Received from Medical College of Virginia, School of Pharmacy, Richmond.

† Deceased September 29, 1961.

[1] Professor J. E. Wertz, as quoted from the preface of "The Determination of Molecular Structure," by P. J. Wheatley, Oxford University Press, London, 1959.

[2] It will be out of the question to give for this review a complete bibliography. The interested student will find it rewarding to examine these early developments of the concepts of chemistry in three dimensions as reported in the biographies of men prominent in this denouement (1–6).

[3] Pasteur himself has given a description of this dramatic moment, "Alors, trés visiblement ému, l'illustre vieillard me prit le bras, et me dit, "Mon cher enfant, j'ai tant aimé les sciences dans ma vie que cela me fait battre le coeur" (Ref. 1, p. 326).

[4] Today's scientist is amazed at the clarity with which Pasteur perceived a century ago the broader implications of such asymmetry, "At dès lors si l'influence mystérieuse à laquelle est due la dissymétrie des produits naturel venait a changer de sens ou de direction, les éléments constitutifs de tous êtres vivant prendraint une dissymétrie inverse. Peut-être un monde nouveau s'offraint a nous. Qui pourrait prévoir l'organisation des êtres vivant, si la cellulose de droite qu'elle est devenait gauche, si l'albumine du sang de gauche devenait droite? Il y a là, des mystères qui préparent à l'avenir d'immenses traveaux et appelent des aujourdhui les plus sérieuses meditations de la science" (Ref. 1, p. 338).

[5] Ostwald (Ref. 3, p. 2224) observed, "Es heisst . . . vielmehr deutlich zu machen . . . dass der Gedanke damals in der Luft lag . . . in welchen ich zu meinen wissenschaftlichen Arbeiten vorbereitete, gelegentlich auch von Strukturformeln im Raume die Rede war."

[6] The geometry of the carbon atom may be comprehended by considering the four valences as terminating at opposite corners of a cube. The large angle of an isosceles triangle, whose legs are half the diagonal of the cube and the third side is the diagonal of the face of the cube, is the angle of the valence bonds.

With the "tetrahedral" character of the carbon atom thus firmly established, students pursuing these problems could now explore the limits of these phenomena, collect and correlate new information, and strive for precise terminology.

NOMENCLATURE

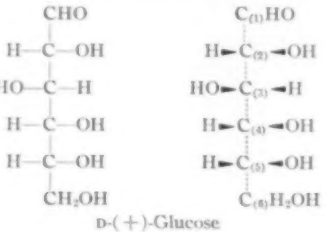
The geometry of three dimensions is well known; nevertheless, in discussing the structures with dimensions of molecules, that is, measured in ten millionths of a centimeter (1 Ångström unit = 1×10^{-8} cm.) precise definition becomes important and unequivocal terminology indispensable. Atoms cannot be seen, and when "models" are introduced, which at best leave much to be desired, sometimes including too much and at other times too little, the situations already exist which can lead to misunderstanding. It is little wonder that during the decades following van't Hoff and LeBel there developed confusion in terminology, some of it plaguing us even today. The principles of stereoisomerism depending on the asymmetric carbon atom were, perhaps, better understood from the first than there was agreement on terms for defining these concepts. Fortunately, today the scientist has the benefit of "official" or generally adopted rules of nomenclature which are doing much to eliminate double talk, at least for the simpler enantiomorphs.[7]

Relative Configuration.—Emil Fischer was unable to determine the exact configuration of an asymmetric carbon atom, but feeling an urgent need for some term of reference, he adopted $C_{(5)}$ of D-glucose as a point of departure. He degraded this sugar to an aldotriose, glyceraldehyde, in which a single stereogenic center of the original glucose remained; he arbitrarily assigned to this product the name and structure indicated. All substances

D-(+)-Glyceraldehyde L-(−)-Glyceraldehyde

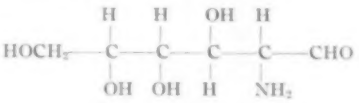
which could be configurationally correlated with dextrorotatory glyceraldehyde were assigned by Fischer to the D-series, and by the same token, those that were correlated with L-glyceraldehyde were placed in the L-series. As a simple example, take the glyceric acids. The acid obtained by the oxidation of the *dextro*-aldehyde was *levo*-rotatory, but since there was no modification of the geometry of the asymmetric carbon atom, the product was called D-(−)-glyceric acid, the D- showing its relationship to $C_{(5)}$ of D-glucose and the symbol (−)- showing the direction in which plane polarized light is turned by a solution of the acid.

Out of modest and at times nebulous beginnings grew conventions which are exemplified in the formulas shown herewith. The "aldehyde" structure for D-glucose, it will be observed, has at $C_{(5)}$ the configuration for D-glyceraldehyde.

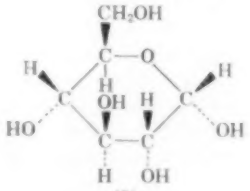


D-(+)-Glucose

The heavy lines indicate that the groups are forward from the plane of the paper; or as in the formula at the left, the horizontal substituents are forward; the dotted lines, or merely the vertical groups, are "back" of the paper. Formulas may also be written horizontally, in which case "the top orienting group, $C_{(1)}$, lies to the extreme right" (8). The formula for D-glucosamine, for example, may properly be written as[8]



For the hemiacetal or cyclic formulas the cycle is considered as substantially planar, and the substituents are written as "up" or "down"

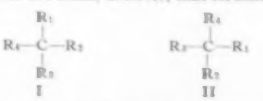


(I)

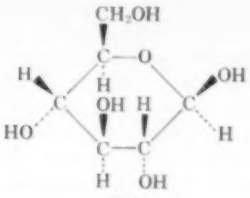
α-D-Glucose

[7] One may feel at first glance, for example, that precise reporting as that of Wolfrom and co-workers (7) is needlessly cautious in correlating the structure of L-(+)-alanine with D-glucosamine. Yet papers like this serve to emphasize the need for a particularized vocabulary for stereochemistry.

[8] This does not mean, however, that formulas I and II are

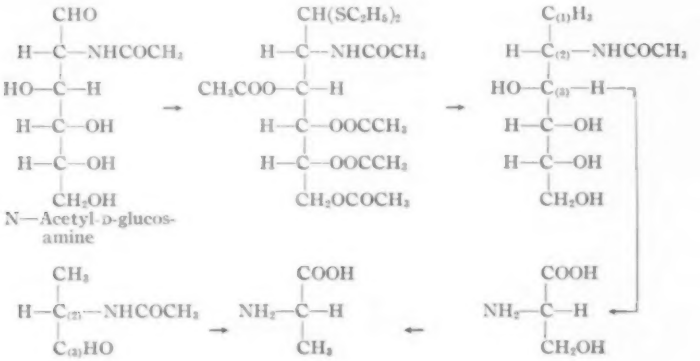


equal in the sense that the rotation of I through an angle of 90° affords II. Rather, the two formulas are enantiomorphic, for by convention groups R_2 and R_4 are "forward" in I and "back" in II.

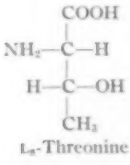


(II)
β-D-Glucose

For α-amino acids, natural serine is the reference standard; its configuration has been correlated unequivocally with that of the isomeric glyceraldehydes. One of these correlations has been contributed by Wolfrom and co-workers (7); it depends on the following:[9]



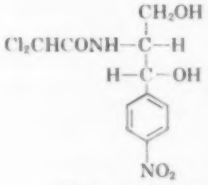
A structure for which *a priori* either the carbohydrate or the amino acid nomenclature may apply requires special attention. L-Threonine, for example, when first characterized and identified as possessing the configuration shown was named in the sugar series and originally called D-threonine.



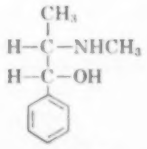
L_g-Threonine

Subsequent redefinition and assigning as an α-amino acid requires L_s-threonine (the subscript s indicating serine series) (9, 10).[10] In the carbohydrate terminology, L_g-threonine becomes 2-amino-2,4-dideoxy-D_g-threonic acid (the subscript g indicates carbohydrate series).[11]

Further examples of terminology are indicated for chloramphenicol and ephedrine.



Chloramphenicol
D_g- (or L_s)-*threo*-1-*p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol



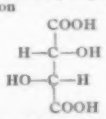
Ephedrine
D_g- (or D_s)-*erythro*-1-phenyl-2-methylamino-1-propanol

Relative *vs.* Absolute Configuration.—Fischer, as already mentioned, adopted $C_{(5)}$ of natural glucose, or the single stereogenic car-

[9] In these and subsequent correlations, not all the intermediate chemical steps in the interconversions will be indicated.

[10] This change in name must be borne in mind for anyone checking the early literature on this essential amino acid.

[11] It may not be amiss to give further examples of the problems for precise nomenclature of stereoisomers. Natural, *dextro*-rotatory tartaric acid is variously named as D-(+)-tartaric acid, L-(−)-tartaric acid, and in a chemical encyclopedia (11) the same formula within a space of three lines is called both D-(+)- and D-(−)-tartaric acid. The natural, dextrorotatory acid is shown by degradation to D-lactic acid, to have the configuration



For further discussions and examples of inconsistencies in nomenclature of this simple substance, attention is directed to four recent papers (12–15).

bon atom of D-(+)-glyceraldehyde as a point of reference for defining optically active compounds. His assignment of configuration, though purely arbitrary at the time, now appears to be most fortunate. Beginning in 1949, a group of Dutch workers (16–19) employing X-ray diffraction measurements on sodium rubidium tartrate and depending on a phase shift which occurs near the absorption edge, have concluded that these conventional formulas also represent the "absolute" structures. X-ray analysis leads to a scaled and geometrically exact structural model in which the interatomic distances may be indicated within the limits of 0.01 Å. Thus, it now becomes permissible to speak of all structures that have been correlated with tartaric acid in terms of absolute configuration.

The rules of nomenclature discussed above are quite universally accepted and serve adequately for carbohydrates and amino acids and even for most acyclic compounds, but they are proving inadequate for the more complex structures, particularly for the polycyclic products with several stereogenic centers. Considerable thought and effort are being given to devising sophisticated rules which are concise, comprehensive, and at the same time, readily understood. The developments along these lines may be gleaned from various reports, e. g., by the Committee on Nomenclature, American Chemical Society, and the proposal of McCasland (20), the recommendations of Cahn, Ingold, and Prelog (21), Cahn and Ingold (22), and others (23). It must be emphasized that these are in the developmental stages and as yet none of the proposals is "officially" approved; it is to be hoped that the cumbersome features which now burden each of them will one day be eliminated, or at least minimized, and that terminology will become standardized and some method merit general approbation. In order to indicate a trend in current endeavors, the following paragraphs describe, in considerably simplified and elementary manner, the ideas advanced by Cahn, Ingold, and Prelog (21).[12]

This method for specifying configurations depends on three steps which may be outlined briefly as follows: (*a*) Each of the four groups attached to the asymmetric carbon atom under consideration is assigned a priority sequence (22). This priority sequence is a function of the "atomic number" of the element substituted on the carbon atom. (*b*) From the priority sequence, a right- or left-hand pattern (clockwise or counterclockwise) is determined. (*c*) Once this pattern is established, a configurational label is assigned to the asymmetric atom. The symbols used are, *R* (from the Latin, *rectus*) for the right-handed pattern and *S* (from the Latin, *sinister*) for the left-handed pattern.

The application of the priority sequence rule may be illustrated with a compound, *Cabcd*, in which the substituents in decreasing atomic number are *abcd*. The molecule is viewed from a direction remote from the lowest, i. e., from *d*; if the plane defined by *a*, *b*, and *c* shows these to be in clockwise sequence, the center is assigned the *R* configuration; if counterclockwise, the *S* configuration is assigned.

These concepts are best visualized with the aid of three-dimensional models, but in the absence of these, perhaps the customary method of representing the tetrahedral carbon by the projection of a tetrahedron will be helpful. The projection is drawn to represent the group with the lowest priority as back of the plane of the paper, thus

I II

In I the priority sequence, $a \rightarrow b \rightarrow c$ is clockwise, hence *R*. In a similar manner, II represents the *S* configuration.

Elementary as these ideas may at first appear, difficulty may be anticipated in their application: (*a*) in translating many conventional stereochemical formulas to show the asymmetric carbon atom in the simple fashion as in I or II above; (*b*) the method becomes cumbersome when many asymmetric centers are present and (*c*) the rules for assigning the priority sequence tend to become involved, since in a large majority of instances other carbon atoms are attached to the stereogenic center. Nevertheless, the system appears to possess advantages which lead to its increasing use.

The sequence rule requires that the groups are to be arranged in order of decreasing atomic number. If it is impossible to choose between two atoms immediately attached to the stereogenic center, then the atoms once removed come into consideration—this process continues until a priority decision can be made.

In molecules of the greatest interest to the medicinal chemist, the elements attached to an asymmetric carbon atom are selected from hydrogen, carbon, nitrogen, oxygen, halogen, and sulfur. Among these, the priority sequence is halogen (except F) $\rightarrow$ S $\rightarrow$ O $\rightarrow$ N $\rightarrow$ C $\rightarrow$ H.

[12] Anyone seriously thinking of employing these rules should be guided by the complete and detailed publications.

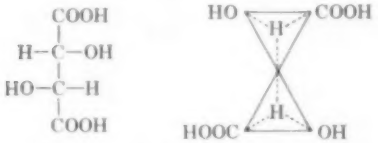
Among the once removed considerations are found, in general, the following sequence: —C—X → —C—S → —C—O (—COOH → CHO → —CO^-) → C—N → —$C(C)_3$ → —$C(C)_2$ → —$C(C)_1$ → —C—H.[13]

Examples of the application of the sequence rule in specifying configuration are given in the following, where the projection of a tetrahedron represents the asymmetric carbon atom. For the enantiomeric glyceraldehydes, the hydrogen atom, having the lowest priority, is placed at the apex furthest away from the viewer.

The sequence is read HO → CHO → CH_2OH in a clockwise direction and the configurational symbol is *R*.

The sequence is read HO→ CHO→ CH_2OH in a counterclockwise direction and the configurational symbol is *S*.

For (+)-tartaric acid, we find



HO → COOH → CHOHCOOH
= *R*

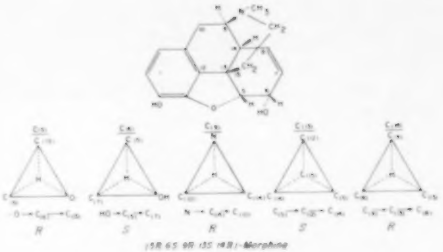
HO → COOH → CHOHCOOH
= *R*

For L-(+)-serine,

NH_2 → COOH → CH_2OH = *S*

The stereochemical formula for morphine has been established as shown in the accompanying formula. Each of the five stereogenic centers is also projected separately as a tetrahedron in order to facilitate description according to the priority sequence rule.

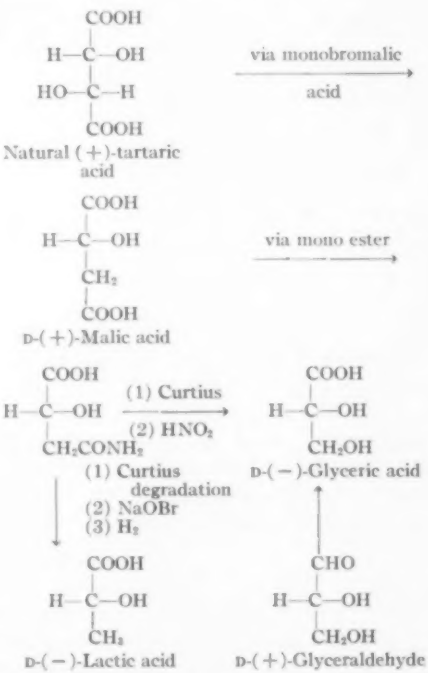
[13] These brief statements lack many significant details for which the original rules of priority sequence should be consulted (22).



METHODS FOR CORRELATING STEREOCHEMICAL CONFIGURATIONS

The procedures for establishing configurations may be summarized and illustrated under the following headings:

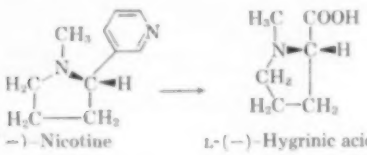
Conversion to D-Glyceraldehyde (or Its Enantiomer) or to a Compound Already so Correlated.—In this method no bond to the stereogenic carbon atom is disturbed, although the substituents on that carbon may be modified as necessary. This procedure is decisive. An example of such correlation is shown by the following sequence of formulas:



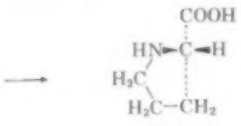
Originally this correlation defined the structure of (+)-tartaric acid relative to D-(+)-glyceraldehyde. Now that the absolute configuration for tartaric acid has been confirmed as being correctly

represented, this correlation serves to establish the absolute structure of D-glyceraldehyde, (+)-malic acid, D-(−)-lactic acid, and so on.

For nicotine (24), the correlation is confirmed as follows:

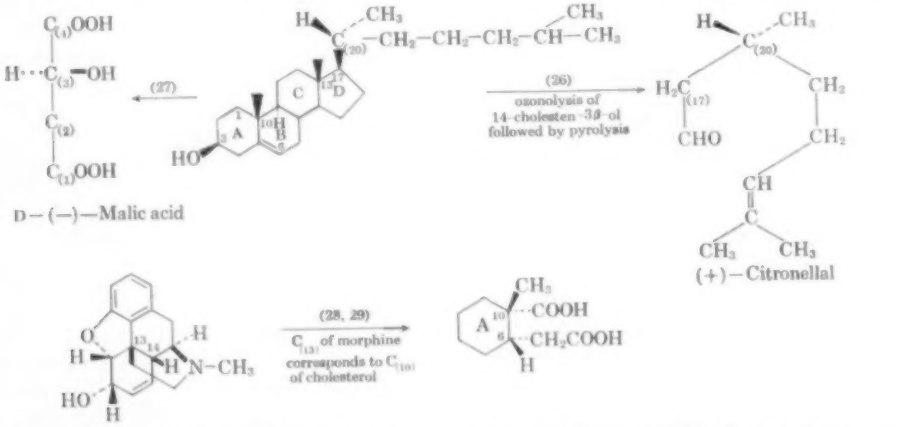


(−)-Nicotine L-(−)-Hygrinic acid



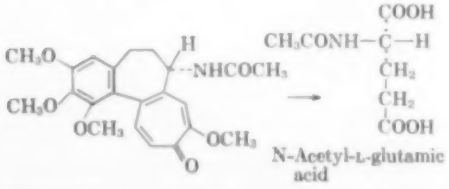
L-Proline

Such correlations also provide information about more complex natural products. For the phenanthrenes, for example, the various steroids[14] and opium alkaloids, once the molecular architecture was established, the configurations were established in part by the following conversions:



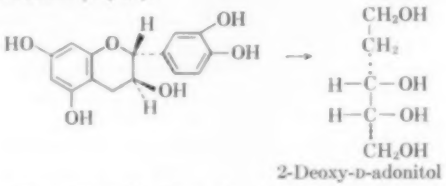
D-(−)-Malic acid (+)-Citronellal

Colchicine, in which the acetamido-bearing carbon atom alone is stereogenic, has been degraded to N-acetyl-L-glutamic acid, thus leading to the following formula for the alkaloid (30)



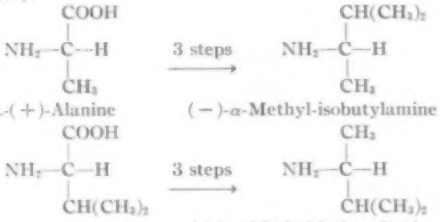
N-Acetyl-L-glutamic acid

A key to the stereochemistry of the flavanoids appears in 2-deoxy-D-adonitol, which has been correlated with a degradation product from (+)-catechin (31, 32).



2-Deoxy-D-adonitol

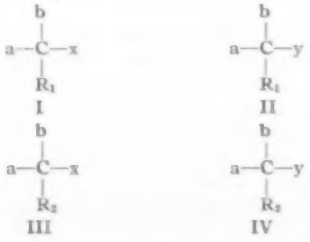
The series identity of L-(+)-alanine and L-valine was confirmed by their transformation by similar reactions into enantiomorphic amines (33).



L-(+)-Alanine (−)-α-Methyl-isobutylamine

(+)-α-Methyl-isobutylamine

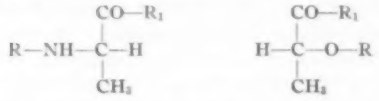
The Displacement Rule, Verschiebungssatz (34), the Rule of Optical Superposition (35).— These principles depend on the following observations. Given four compounds of general structure (36)



I II III IV

[14] A complete discussion of the configuration in the steroid series is given by Fieser and Fieser (25). For rules of nomenclature, see (10).

which are configurationally related, the replacement of R_1 by R_2 in I and II affording III and IV, respectively, will manifest itself by a change in rotation, but the shift will be in the same direction, and the greater the similarity between R_1 and R_2 the nearer in magnitude will the shifts be. A similar situation obtains in substituting y for x. Thus, if the rotational shift of both for I → II and III → IV are in the same direction, they are again shown to possess identical configurations. Thus, for example, the correlation between L-(+)-alanine and D-(−)-lactic acid was made by comparing derivatives of types

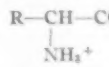


modifying in single steps R from H— and R_1 from —OH in both structures; in every instance, the shift was observed to be in opposite directions.

By application of these principles, it has been possible to place all the α-amino acids obtained from protein hydrolysates in the L-series (*cf.* Neuberger, *loc. cit.*). Special application of the principle is seen in Hudson's rules of isorotation, the amide rule, the phenylhydrazide rule, and the lactone rule (37). An instance of erroneous conclusions by the use of these methods is described by Hirst (38).

Physical Methods.—Similarly constructed molecules of identical series undergo like shifts in rotation when subjected to identical changes, e. g., of temperature, dilution, addition of neutral salts, complex formation, etc. (39, 40) (*cf.*, Neuberger, *loc. cit.*, p. 300).

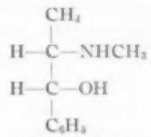
The specific rotations of solutions of asparagine, aspartic acid, glutamic acid, leucine, serine, and alanine become more *dextro* on addition of mineral acid owing to the formation of the ion



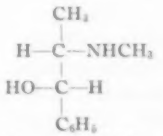
On the basis of these observations, Clough (41) concluded that all amino acids showing parallel shifts must be configurationally related. Levene and co-workers (42–44) extended these studies. Leithe (45) determined that (+)-pipecolic acid is D-. du Vigneaud and Irish (46) assigned (+)-2-amino-4-phenylbutyric acid to the L series. The influence of solvent and salt-formation on the rotatory properties of (−)-laudanosine led Leithe (47) to conclude that the asymmetric center of the alkaloid is to be correlated with the L-amino acids; this conclusion is supported by data observed for the L-(−)-α-phenethylamine (48).

Hawthorne and Cram (49) have demonstrated that it is possible to use differences in the ultraviolet absorption spectra of certain diastereomerically related compounds to determine the relative configuration of the stereogenic centers involved. Their correlations are based on the observation that steric inhibition of resonance in the systems they employed decreases the intensity and generally the wavelength of the major absorption peak exhibited by the system. Wepster (50) has reviewed some aspects of this phenomenon.

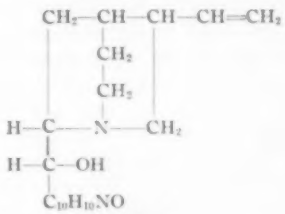
By Analogy.—This method has much in common with the preceding. The assignment of configuration to the cinchona alkaloids has been made by comparison with ephedrine and pseudoephedrine (51).



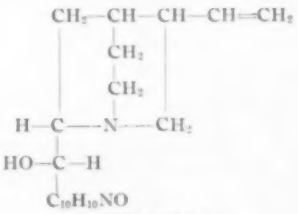
Ephedrine



Pseudoephedrine



Quinine



Epiquinine

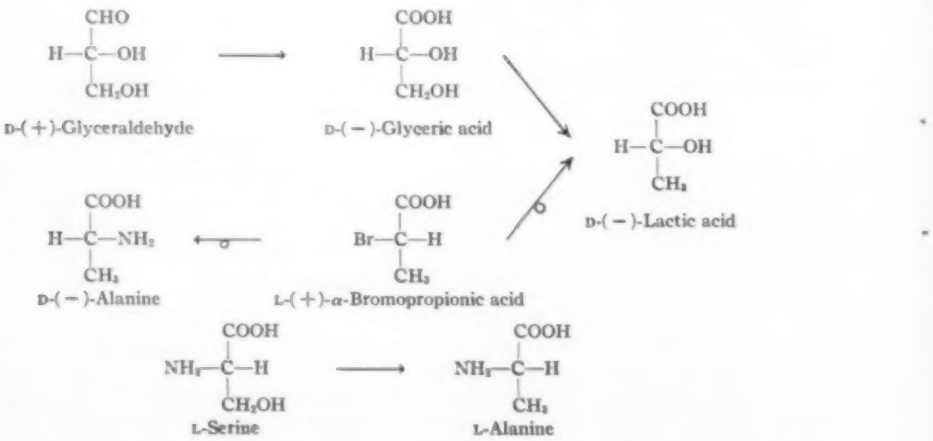
Quinine and epiquinine are epimeric at the hydroxyl-bearing carbon atom, just as are ephedrine and pseudoephedrine. Pseudoephedrine, the threo configuration, is a stronger base than ephedrine, the erythro-isomer; epiquinine is a stronger base than quinine and thus, by analogy,

is assigned the indicated structure, and quinine is then written as the erythro-isomer (with respect to positions 8 and 9). Quinidine is the mirror image of quinine only at $C_{(8)}$ and $C_{(9)}$; positions 3 and 4 have identical configurations as indicated in all the cinchona alkaloids and in cupreine.

Similar analogies were employed in determining the configuration of natural chloramphenicol (52). The stability of the base under alkaline and acid hydrolysis and various acylation conditions, as well as the rotation values of the base and its derivatives, indicates, by analogy, that chloramphenicol is sterically classified in the more stable pseudoephedrine series. Further, the sign of the optical rotation of the antibiotic, its derived base, and its *p*-nitrobenzamide indicated it to be a derivative of (−)-norpseudoephedrine. The data are given in Table I.

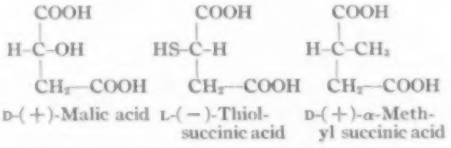
The most probable structure for chloramphenicol, as indicated by these data, is D-(−)-*threo*-2-dichloroacetamido - 1 - *p* - nitrophenyl -1,3-propanediol.

Application of Established Reaction Mechanisms.—A knowledge of stereochemistry has been of inestimable value in studying the reaction mechanisms of organic chemistry, and now that these are better understood, their employment is helpful in establishing and confirming stereochemical structures. The replacement of the bromine atom, for example, in L-(+)-α-bromopropionic acid under kinetically controlled conditions which bring about an S_N2 reaction is accompanied by inversion as indicated (53)[15] and the correlation to glyceraldehyde is shown:[16]



It is known that the presence of an asymmetric atom within a molecule will influence the configuration assumed by a newly created center, thereby determining the ratio of isomers formed. Cram and Abd Elhafez (55) have accordingly formulated the "Rule for Steric Control of Asymmetric Induction," which has been used to predict on the basis of steric factors, the configuration of the product. It has been applied most often to products obtained from ketones with the carbonyl α to an asymmetric center; an entering group, say, from a Grignard reagent, approaches the ketone from the least-hindered direction, and thus one may predict whether the erythro or the threo configuration predominates in the product.

Formation of Quasi-racemates (56).—Two optically active compounds of very similar but of opposite configuration mixed in equimolar quantities show melting point behavior characteristic of racemates. Thus, (+)-malic acid and (−)-thiolsuccinic acid are shown to be of opposite configuration; and (+)-methyl succinic acid forms a quasi-racemate with (−)-thiolsuccinic acid, since the configuration of (+)-malic acid is known, the following configurational relationships are indicated:

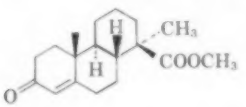


Rotatory Dispersion.—The change in optical rotation with change in wavelength of light was first observed in 1817 by Biot. By plotting the optical rotation of a compound on one axis and the wavelength of light on the other, a

[15] In dilute alkali, first-order hydrolysis of the α-bromopropionate ion has been established since the lactate ion is formed with retention of configuration (54).

[16] The sign $\xrightarrow{\text{O}}$ signifies inversion.

rotatory dispersion curve is obtained. Owing to the convenient access to monochromatic light, the D-line of sodium, the study of rotatory dispersion lay comparatively neglected until 1953, when Djerassi and co-workers (57) initiated a study of these phenomena. In a short space of time they had measured some 2,000 complex natural products or their chemical modifications. First studied were those of known configuration in order to collect data for establishing reliable generalizations or rules, and then were measured substances of unknown configuration. These investigations are proving specially useful for polycyclic steroids and higher terpenoid products. As one example, 8,13-dimethyl-8-methoxycarbonyl - 2 oxo - $\Delta^{1(11)}$ - dodecahydrophenanthrene (structure given), derived from neoabietic acid, shows a multiple Cotton effect curve very similar to that of 19-nortestosterone (of known configuration) and therefore, the stereo-structure as given,



is assigned to the product; were it an enantiomorph, the dispersion curves would have been of the mirror-image type. It can be easily understood that such techniques must be of invaluable assistance in complex substances with multiple stereogenic centers, e. g., in the cholestane structure there are eight neighboring asymmetric carbon atoms, *viz.*, in the following sequence, 5-10-9-14-13-17-20.

Enzymic Procedures.—The first observation of record that enzymes react selectively or preferentially with one isomer of enantiomorphic pairs was made by Pasteur (58) when he found that a solution of racemic ammonium tartrate supporting the growth of *Penicillium glaucum* first became *levo*-rotatory, indicating metabolism of the *dextro*-rotatory components. A systematic study of these phenomena was made by Fischer (59); extending his observations, he was able, for example, to distinguish between α- and β-glycosides, the former being hydrolyzed by yeast and the latter by emulsin.[17]

The preferential utilization of L-amino acids by microorganisms was first observed in 1886 by culturing *Penicillium glaucum* on racemic acids and it was possible to isolate the slowly metabolized D-α-amino acids (60). Ehrlich (61) took advantage of this preference of the organism for a single enantiomer and from racemic substrates was then able to isolate up to 70 per cent pure D-amino acids; even of such compounds as D-α-phenylglycine, of which the L-isomer does not appear in natural products. By growing yeast on racemic hexoses, Fischer (62) found that the natural isomer was metabolized, leaving the unnatural configuration for isolation from the residue.

It is now known that there are stereospecific enzymes, such as L-amino oxidases, D-amino acid oxidases, L-decarboxylases, L-acylases, L-amidases, and that these will react upon suitably substituted α-amino acids but only if the configuration is favorable. Hence, appropriate use of such enzymes affords information which may be employed for assignment of correct configuration (63).

By means of renal acylases, Greenstein and co-workers (*loc. cit.* p. 172), found that for some 45 N-acylated racemic acids, the L-components were deacylated, that is resolving the racemic product into pure unchanged D-amino acid derivative and pure L-amino acid, which could be conveniently separated. Since the D-derivative could be hydrolyzed by chemical means, the mediation of the enzyme provided not only excellent resolution but reliable information as to the series configuration.

Stereoselective Adsorbents.—A stereoselective adsorbent exhibits higher capacity for isomers of related configuration, provided their structures are not too dissimilar, than for their diastereoisomers. Thus, Beckett and Anderson (64) found that silicon dioxide precipitated in the presence of quinine and the alkaloid then removed completely adsorbed cinchonidine more readily and to a greater extent than did a gel similarly prepared with quinidine; the quinidine gel, however, favored the adsorption of cinchonine.[18]

Stereospecific Genesis.—The propagation of life and the continuation of the species de-

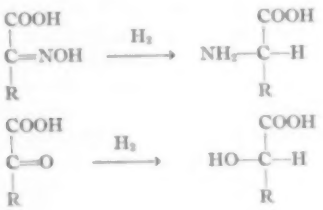
[17] Fischer's analogy of these enzymes to a lock-and-key mechanism is given in the following words (*loc. cit.*, p. 2992): "Invertin und Emulsin haben bekanntlich manche Aehnlichkeit mit den Proteinstoffe und besitzen wie jene unzweifelhaft ein asymmetrisch gebautes Molekül. Ihre beschränkte Wirkung auf die Glucoside liesse sich also auch die annahme erklären, dass nur bei ähnlichen geometrischen Bau diejenige Annäherung der Moleküle stattfinden kann, welche zur Auslösung des chemischen Vorganges erforderlich ist. Um ein Bild zu gebrauchen, will ich sagen, dass Enzym un Glucosid wie Schloss und Schlüssel zueinander passen müssen, um eine chemischen Wirkung aufeinander zu üben können."

[18] The problem of stereospecific adsorption would appear to be worthy of more attention than it has received thus far. Reports, for example, that wool preferentially adsorbs one isomer of a racemic dye (65), that wool and casein selectively adsorb (+)-mandelic acid, and (+)-naphthylglycolic acid (66) and that racemic Tröger's base was resolved chromatographically by passing over lactose monohydrate (67) support this suggestion. Further indication that stereospecificity may be important in adsorptive mechanisms appears in the hypothesis of Beckett and Casy (68) that analgesics such as methadone and morphine, with a configuration complementary to the L-amino acids, react more favorably with the receptor sites in the tissues.

TABLE I.—CHLORAMPHENICOL AND PSEUDOEPHEDRINE SERIES DATA

	(−)-Norpseudoephedrine		(−)-Norephedrine		Base Derived from Chloramphenicol	
	M. P., °C.	$[\alpha]_D^{27}$	M. P., °C.	$[\alpha]_D^{27}$	M. P., °C.	$[\alpha]_D^{27}$
Base	77–78	−32.64	50	−14.56	162.3	−23.1
Base·HCl	180–181	−42.68	171–172	−33.27	210–211	−76.9
N-*p*-$NO_2C_6H_4CO$-	199	−105	175–176	−49.6	204.5	−140.2

pends on the natural generation of "*dextro*-celluloses and *levo*-albuminoids" to use the words of Pasteur. This process is, of course, one of the miraculous phenomena of all life. The organism does not produce falsely, and it "knows," as it were, the configuration of its products. The study of stereochemistry is, in a large measure, an effort to learn about these biogenetic pathways and the configurations of the multifarious products. This being true for life processes, it would seem possible, *a priori*, that a stereospecific nonenzymic catalyst, for example, an L-genic catalyst, suitable symmetrical substrates would be stereospecifically modified into products of identical series. For example, with an effective L-genic hydrogenation catalyst, one might anticipate reactions such as

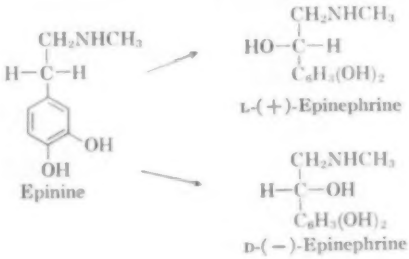


This prospect is not just pure theory, for Beamer and co-workers (69) have provided evidence in support of substrate-specific and also stereospecific centers in palladium-on-carbon catalysts. Furthermore, they, along with Smith (70), have prepared catalysts in which the hydrogenation of symmetrical substrates produces one enantiomorph in larger amount than its isomer. These catalysts were obtained by appropriate selection of carrier, e. g., by precipitating silica in the presence of quinine, or by coating, say, charcoal carrier with optically active amino acids before deposition of the palladium.

STEREOCHEMISTRY IN BIOLOGICAL SYSTEMS

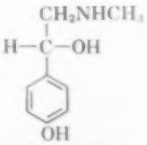
Pasteur's discovery of the preference of *Penicillium* for D-tartrates marks the beginning of our appreciation that stereoisomerism plays a significant role in biological processes. The scientific literature is replete, a century later, with uncounted examples of similar import. Although it is beyond the scope of this review to list them, it will be profitable to look into some of them more closely in order to give emphasis to the scope and breadth of these phenomena.

Compare, for example, D-(−)-epinephrine and L-(+)-epinephrine; the former may be looked upon as epinine, a symmetrical pressor amine, structurally modified into a D-carbinol, and the latter into the enantiomeric L-carbinol as indicated in the respective formulas



These three compounds were first studied by Barger and Dale and the comparisons for pressor activity are reported in their classical paper, the first to give any comparison of chemical structure changes with pharmacological activity (71). And they have been studied many times since then only to confirm the results first observed by this distinguished pair of investigators. We are now able to conclude on the basis of all these studies that the conversion of epinine into L-epinephrine is accompanied by a decrease in the pressor activity to about one-third that of epinine; and the conversion to the D-isomer is accompanied by about a twelvefold increase in pressor activity.

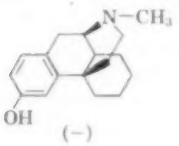
For synephrine, Tainter observed not only quantitative differences but qualitative differences as well. But more than that, the



(+)-synephrine neutralizes or antagonizes the ability of the (−)-isomer to produce vasoconstriction when perfused through the rabbit ear (72, 73).

As further examples, mention may be made of the availability as metabolites of the L-α-amino acids, e. g., thyroxine (74), L-tryptophan (75); or the inability of D-thienylalanine to function as an antimetabolite as does the L-thienylalanine (76). Berg (77) has compared the D- and L-α-amino acids with regard to taste, enzymatic hydrolysis of derivatives, gastrointestinal absorption, and other properties.

(−)-N-Methyl-3-hydroxymorphinan is more potent as an analgesic (and also in its addictive properties) than natural morphine; (+)-N-methyl-3-hydroxymorphinan



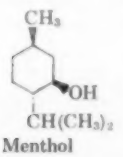
is neither addictive nor analgesic, but it does retain the antitussive ability of the opium alkaloids (78).

Quinine is accepted by U.S.P. as an antimalarial agent, but the stereoisomeric quinidine is listed as an antifibrillatory agent (79).

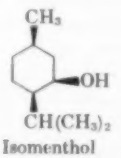
D-Ascorbic acid, though quite as effective as the natural L-ascorbic acid in the biochemical oxidation of tyrosine (80), is completely ineffective in doses up to 20 mg. daily, or forty times the active dose for L-ascorbic acid, when tested on ascorbutic guinea pigs (81). L-Ascorbic acid persists in the guinea pig, half of the amount still being present after three to four days; the D-isomer is rapidly metabolized and excreted in twelve hours or less (82).

Riboflavin, vitamin B_2 or G, serving as a biological transporter of oxygen, is extremely stereospecific. Epimerization at only one point, e. g., as in D-araboflavin, affords a diastereoisomer with antagonistic properties (83).

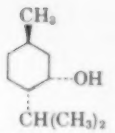
Only menthol (I) possesses the agreeable odor of peppermint. Isomenthol (II) and neomenthol (III) are musty and disagreeable, and neoisomenthol (IV) is even more objectionable.



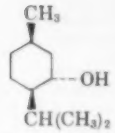
Menthol
I



Isomenthol
II

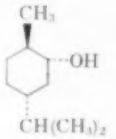


Neomenthol
III

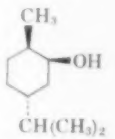


Neoisomenthol
IV

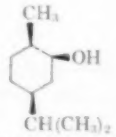
Analogous results are observed for the carvomenthols, carvomenthol (V) being most like menthol and neoisocarvomenthol (VII) being nauseating (84).



Carvomenthol
V

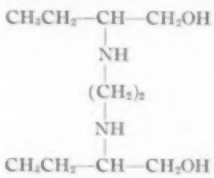


Neocarvomenthol
VI



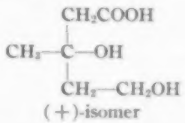
Neoisocarvomenthol
VII

2,2′-(Ethylenediimine)-di-l-butanol, is a synthetic antitubercular agent.



The (+)-isomer, which shows activity four times greater than does streptomycin, is twelve times more active than the meso-, and two hundred or greater times more active than the (−)-isomer; the acute toxic dose is about the same for the (+)-, (−)-, meso-, and racemic modifications (85).

Mevalonic acid of structure



(+)-isomer

is an intermediate in the biosynthesis of terpenes and steroids (86). The inability of the organism to use the enantiomer was determined by employing racemic mevalonic acid; as the (+)-isomer was consumed, the (−)-isomer remained.

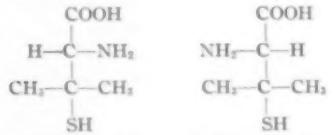
The D-isomers of thyroxine and triiodothyro-

TABLE II.—D-(−)- AND L-(+)-MANDELATES OF D-(−)- AND L-(+)-NOREPHEDRINES

Isomer	Symbol	M. P., °C.	$[\alpha]_D$	Solubility at 37°: Gm./10 ml. Water	Gm./10 ml. Saline
D-Norephedrine D-Mandelate	Q_1	171.5–172	−70.6	7.39	7.39
L-Norephedrine L-Mandelate	Q_2	171.5–172	+70.7	8.38	7.34
D-Norephedrine L-Mandelate	Q_3	(Not purified)	...	...	...
L-Norephedrine D-Mandelate	Q_4	164.5–165	−42.8	10.69	10.19

nine lower cholesterol levels in the blood without being over stimulating (87).

Natural penicillins, which on hydrolysis afford D-penicillamine, are active antibiotics. Synthetic penicillins prepared from the same D-α-amino acid are equally effective. Penicillins prepared from racemic



D-Penicillamine L-Penicillamine

penicillamine are half as active, and those obtained with L-penicillamine are practically inactive (88).

The significance of stereochemical reactions, and here must be included the *in vivo* or protoplasmic as well as the *in vitro* reactions, for science may be simply comprehended in the following general statements or equations.

$$D—A + B \rightarrow P_1 \qquad \text{(Eq. 1)}$$

This means that a D-reagent reacts with a symmetrical reagent and the product is P_1.

$$L—A + B \rightarrow P_2 \qquad \text{(Eq. 2)}$$

The enantiomorph of D-A, or L-A, reacts with the same symmetrical reagent and the product P_2 is a stereoisomer of P_1.

$$D—A + D—C \rightarrow Q_1 \qquad \text{(Eq. 3)}$$

Two chemicals, both of the D-series, react to give rise to product Q_1.

$$L—A + L—C \rightarrow Q_2 \qquad \text{(Eq. 4)}$$

Two chemicals, both of the L-series, and both enantiomorphs of the respective agents shown in Eq. 3, give rise to product Q_2, which is an enantiomorph of Q_1.

$$D—A + L—C \rightarrow Q_3 \qquad \text{(Eq. 5)}$$

One agent of the D-series reacts with a reagent of the L-series, giving product Q_3, which is diastereomeric with Q_1 and Q_2.

$$L—A + D—C \rightarrow Q_4 \qquad \text{(Eq. 6)}$$

The enantiomers of the reagents indicated in Eq. 5, react to give product Q_4, which is the enantiomorph of Q_3 and diastereomeric with Q_1 and Q_2.

It becomes obvious that we may also meet with combinations of the above statements, e. g., Eqs. 3 and 5, which will account for the reaction of an optically active with a racemic reagent, which in effect becomes

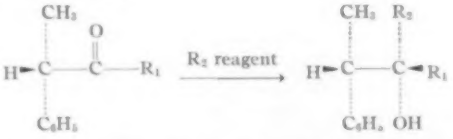
$$D—A + DL—C \rightarrow Q_1 + Q_3 \qquad \text{(Eq. 7)}$$

and for the Eqs. 4–5 combination, we get

$$DL—A + L—C \rightarrow Q_2 + Q_3 \qquad \text{(Eq. 8)}$$

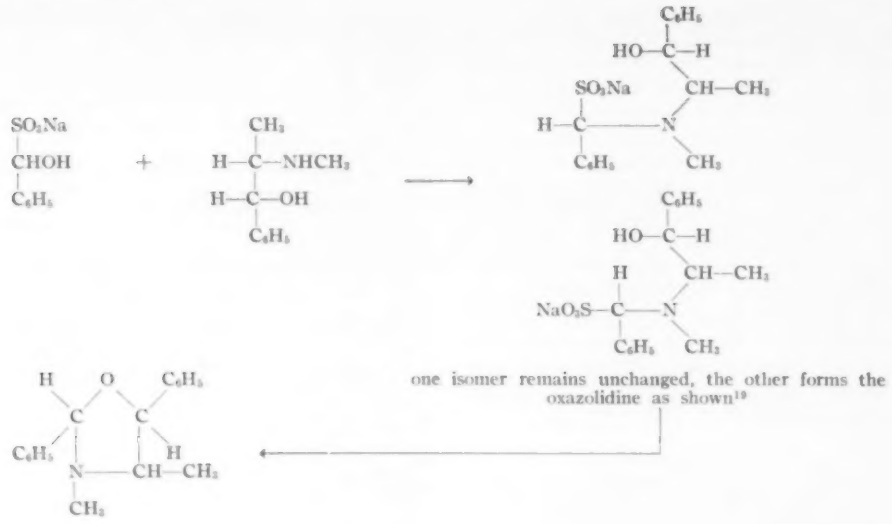
A closer look at these expressions is in order.

Equations 1 and 2 are of fundamental importance in that a study of such systems affords valuable information about the steric influence on reactions. Such information is of utmost value in shedding light on mechanisms. As a simple illustration, the work of Cram and Elhafez (55) may be cited. From a study of the reaction summarized as



they found that the R_2-group entered the carbonyl reagent most readily from the least-hindered direction. Thus, if R_1 = H and R_2 = CH_3-, the product will be predominantly erythro (with respect to both the methyl groups and the hydrogen atoms); if R_1 = CH_3- and R_2 = H, then the product is predominantly threo. Here, then is a simple *in vitro* example of the manner in which the conformation or the configuration of a chemical reagent influences the direction and the extent of the reaction in which it participates. It may be expected that *in vivo* reactions, which are more involved, may be even more sensitive to stereospecific influences.

Neelakantan (89) has also studied a reaction that is somewhat more complex in that two diastereomeric products are formed as in Eq. 7, of which one readily undergoes further modification; the other is presumably formed with such configuration that is unsuitable for this further change. D-(—)-Ephedrine reacts with benzaldehyde-bisulfite at pH 7+ to produce an amino sulfonate as follows:



The formation of the oxazolidine from one of the isomers of the first reaction step is but one indication that the abbreviated statements above are for many cases far too elementary. Nevertheless, in terms of, say, Eq. 7, product Q_1 possesses the structure and conformation to react further, a feature lacking in Q_2. In such terms, it is possible to account for the differences in biological properties of stereoisomers.

For the other four statements, those dealing with the formation of the Q-product, it should be noted that (*a*) Eqs. 3 and 4 are alike in all respects except two. The rates of reaction are identical, all energy requirements are equal, the products Q_1 and Q_2 are physically and chemically alike except that they rotate plane polarized light in opposite directions, though in equal degree, and they differ in contact with other optically active systems. The salts formed from D-(−)- and L-(+)-mandelic acids and D-(−)- and L-(+)-norephedrines are examples of such isomeric products (90), as shown in Table II.

Q_1 and Q_2 mixed in equimolar quantities will form a racemate, an externally compensated product.

(*b*) Similar conclusions hold for Eqs. 5 and 6 and for products Q_3 and Q_4, respectively. However, the Q_1–Q_2 racemate may differ widely and in many respects from the Q_3–Q_4 racemate; these differences may be manifested physically, chemically, and biologically.

(*c*) The dynamics for Eqs. 3 and 4 are identical, as are also those for Eqs. 5 and 6. However, this is most significant, Eqs. 3 and 4 will differ appreciably from Eqs. 5 and 6 in forces within and between molecules, kinetics, mechanism, and energy requirements. It is this fact which accounts for the differences, say, between Eqs. 4 and 6 in explaining why Ehrlich (*loc. cit.*) and others were able to isolate D-amino acids by allowing enzyme to react with racemic modifications of the substrates. In this instance, the enzyme, represented by L-A and the racemic substrate by DL-B becomes a more particularized reaction which may be written as

L-enzyme + L-amino acid → rapidly metabolized (Eq. 4)

L-enzyme + D-amino acid → very slowly metabolized, if at all (Eq. 6)

At times these reactions are extremely specific, particularly in the area of enzymes. Many esterases, for example, will hydrolyze acetylcholine as well as other esters of choline, but true choline esterase will react only with acetylcholine (91).

If, for example, the protoplasmic reaction of riboflavin is represented by, say, Eq. 5, then with D-araboflavin, it becomes modified to

$$D—A' + L—C \rightarrow Q_3'$$

In this instance, we have no idea how nearly Q_3 and Q_3', the immediate products produced by the epimeric flavins are identical, but in any case they are sufficiently unlike to produce profoundly different biological effects.

[19] It is not known which of the amino sulfonates undergoes the cyclization to the oxazolidine, for although it is known that the reaction gives rise to products in which the original aldehyde carbon atom of benzaldehyde becomes stereogenic as indicated, it is not yet known whether the displacement of the sulfonate moiety requires the S_N2 mechanism, that is, is accompanied by inversion.

CONCLUSIONS

About one hundred and fifty years ago, optical phenomena were observed which were to have great impact on the development of science and an understanding of life processes. These phenomena are now explained in terms of the asymmetric, three-dimensional carbon atom. Important as the concepts of stereoisomerism already have been for chemistry, they promise to become even more valuable. They have already clarified many problems in biology, but barely a beginning has been made. With the accelerating pace of science, we may expect even greater enlightenment in the decades immediately ahead. The unsolved problems of stereochemistry are a worthy challenge to the best minds.

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Menispermaceae Alkaloids III

Alkaloids of *Stephania hernandifolia*

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The roots of *Stephania hernandifolia* (Willd.) Walp. from Mangalore, Mysore State, yielded *d,l*-tetrandrine, fangchinoline, *d*-tetrandrine, and *d*-isochondrodendrine. The polymorphism of *d*-tetrandrine and fangchinoline and the identities of "menisine" and "menisidine" are discussed.

STEPHANIA HERNANDIFOLIA (Willd.) Walp. is a menispermaceous slender twining shrub found in India on the west and east coasts, Cachar, Sikkim, East Bengal, and Assam (1). The extract of the plant is piscicidal and strongly poisonous to frogs (1). The roots are reported to have found use in the treatment of fever, diarrhea, dyspepsia, and urinary diseases (2). The plant has apparently also been used on occasion as a source for the Ayurvedic drug, "patha," although *Cyclea peltata* Diels is now regarded as the true source of the latter drug (3).

An earlier examination of *S. hernandifolia* from the eastern coast of Australia revealed the presence of *l*-quercitol and a mixture of alkaloids (4). Tomita and Ueda reported in 1959 (5) on the alkaloids of *S. hernandifolia* purchased as crude drug in the Bombay market. The latter workers isolated isotrilobine, a new tertiary phenolic base, and β-sitosterol. In addition, they reported paper chromatographic evidence for the occurrence of two water-soluble quaternary bases. In 1960, Moza reported that a preliminary study of *S. hernandifolia* from Calcutta showed that the plant contained alkaloids, steroids, and fats (6).

The present report describes a preliminary study of the alkaloids of the roots of *S. hernandifolia* from Mangalore, Mysore State. Coarsely ground plant was extracted successively with petroleum ether, methanol, and 70:30 methanol-water. The extracts were processed for alkaloid content by the procedure summarized in Fig. 1, whereby a 1.0 per cent yield of crude alkaloids was obtained. The crude fractions obtained were either crystallized directly or subjected to chromatography, whereby separation into crystallizable fractions was effected. The isolation procedure afforded *d,l*-tetrandrine (II) as the principal isolable constituent (0.125 per cent) and fangchinoline (I) (0.077 per cent), *d*-tetrandrine (II) (0.071 per cent), and *d*-isochondrodendrine (III) (0.003 per cent) as lesser isolable constituents.

The aforementioned four alkaloids isolable from *S. hernandifolia* have been isolated previously from drugs derived from menispermaceous plants. *dl*-Tetrandrine was recently isolated from *Cyclea peltata* Diels in this laboratory (7). *d*-Tetrandrine was isolated from *S. tetrandra* S. Moore (8), *S. cepharantha* Hayata (9), *Menispermum dauricum* DC. (10), *Cyclea peltata* Diels (7), and from han-fang-chi and mu-fang-chi, obtained from plants in the Mukden area and appearing on the Chinese market (11–14). Fangchinoline has also been isolated from han-fang-chi (12, 14) and from *Cyclea peltata* Diels (7). *d*-Isochondrodendrine was isolated from the drug *Radix pareirae bravae* of the English market (15), from *Chondrodendron platyphyllum* Miers, *Ch. microphyllum* (Eichl.) Moldenke (15), *Ch. tomentosum* Ruiz and Pav. (16), *Ch. candicans* Sandwith (17) and *Ch. limaciifolium* (Diels) Moldenke (18), from *Cissampelos pareira* Linn. (19), from *Cyclea insularis* (Makino) Diels (20) and *Cyclea peltata* Diels (7), and from *Pleogyne cunninghamii* Miers (21).

Menisine and menisidine are names assigned to two alkaloids isolated from mu-fang-chi, possibly derived from *Stephania tetrandra* S. Moore (22–24). Menisine (m. p. 152°) was assigned an empirical formula ($C_{38}H_{42}N_2O_6$) identical to that of *d*-tetrandrine (m. p. 217°),

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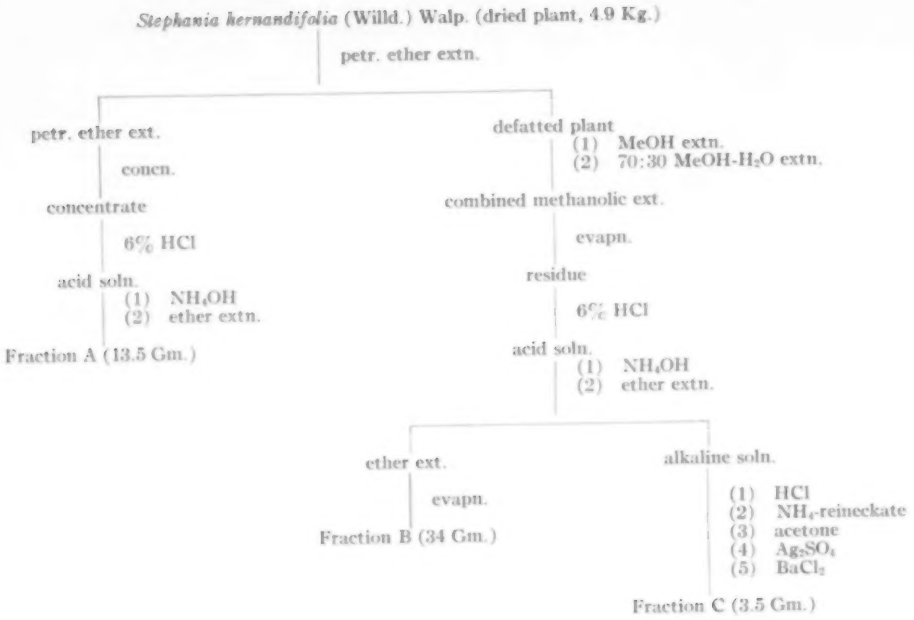
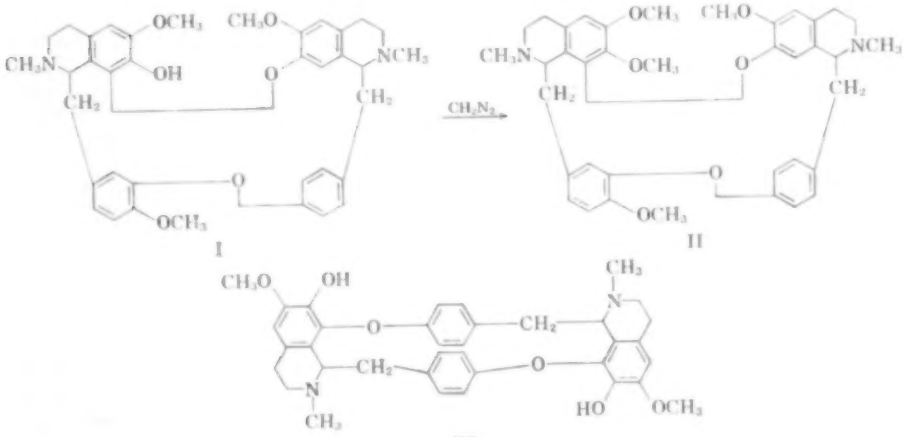


Fig. 1.—Flow sheet for separation of alkaloids of *Stephania hernandifolia* (Willd.) Walp.



and afforded derivatives and degradation products with properties identical with those derived from *d*-tetrandrine (22, 25). When menisine was heated at 150° for three hours, it was transformed into *d*-tetrandrine. Menisidine (m.p. 176°) was assigned an empirical formula ($C_{37}H_{40}N_2O_6$) identical with that of fangchinoline (m. p. 237–238°), was transformed into derivatives with the same properties as the corresponding derivatives from fangchinoline, and was converted to fangchinoline when heated at 160–170° for five hours (22, 26). Furthermore, methylation with diazomethane converted menisidine to menisine (26). Menisine and menisidine were found to possess pharmacological properties similar to those of *d*-tetrandrine (27). From the data previously recorded in the literature, it appeared likely to us that menisine is an isomorphic form of *d*-tetrandrine, and that menisidine is an isomorph of fangchinoline. We have indeed found that *d*-tetrandrine and fangchinoline each crystallize from different

solvents in different forms. When *d*-tetrandrine (from ether, m. p. 217–218°) was recrystallized from acetone, colorless needles, m. p. 149–150°, were obtained (*cf.* "menisine," m. p. 152°). When fangchinoline (from acetone, m. p. 237–238°) was recrystallized from methanol, prisms, m. p. 177–179°, were obtained (*cf.* "menisidine," m. p. 176°). In view of the foregoing, it is exceedingly probable that "menisine" is a low-melting isomorph of *d*-tetrandrine and that "menisidine" is a low-melting isomorph of fangchinoline.

It is noteworthy that the principal isolable alkaloidal constituents of our sample of *S. hernandifolia* were found to be the same as those of *Cyclea peltata* Diels (7), although the relative proportions of the alkaloids differed. The similarity in alkaloid content supports the assigned close taxonomic relationship between the two plants (28). Furthermore, the aforementioned alternative use of either *Cyclea peltata* Diels or *S. hernandifolia* (Willd.) Walp. as the source of the drug "patha" may be rationalized. The similarity in alkaloid content suggests that the pharmacological properties of the respective preparations may well be qualitatively similar in nature.

EXPERIMENTAL

Melting points have been corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Infrared spectra were determined on a Baird double beam infrared recording spectrophotometer. Ultraviolet absorption spectra were determined in 95% ethanol on a Cary recording spectrophotometer (model 11 MS). Paper chromatography was conducted by the descending technique on Whatman No. 4 paper. The procedure used was essentially that of Moza (6), employing butanol and phosphate-citrate buffer at pH 2.2. Best results were achieved with paper pretreated with phosphate-citrate buffer at pH 3.5 and a development period of sixteen hours in a tank saturated with both phases of the solvent system.

Extraction of Alkaloids from *Stephania hernandifolia*

Separation into Main Fractions.—Coarsely ground *S. hernandifolia*[1] (air-dried roots, 4.9 Kg., gathered in Mangalore, Mysore State in January 1959) was extracted continuously for five days with petroleum ether (Skelly B, b. p. 60–68°) in a Soxhlet-type extractor. The total extract (12 L.) was filtered while hot and the filtrate was concentrated (to 4 L.) under reduced pressure. The solution was extracted with 6% hydrochloric acid (7 L.), and the acid extract was washed with ether, and made alkaline with ammonium hydroxide. The alkaline solution was extracted repeatedly with ether. The combined ether extracts were dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to yield 13.5 Gm. of pale yellow solid (fraction A, see Fig. 1).

The dried marc remaining from the petroleum ether extraction was next extracted continuously with methanol for five days, followed by 70:30 methanol-water for four days. When the extraction was stopped, the extract returning to the pot yielded upon evaporation a residue which did not give a positive test with Mayer's reagent. The combined methanolic extracts were concentrated under reduced pressure to a dark brown semisolid residue. The residue was triturated with 6% hydrochloric acid and the acid triturations were repeated until the extract no longer gave a positive test with Mayer's reagent (total volume, 20 L.). The acid extracts were combined, washed with ether, made alkaline with ammonium hydroxide, and extracted with ether (20 L.). The ether solution was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to yield a solid alkaloidal residue (fraction B, 34 Gm.).

The aqueous alkaline solution was acidified with hydrochloric acid and treated with a saturated Reinecke salt solution. The dried reineckate salt was dissolved in acetone and treated with saturated aqueous silver sulfate solution to complete precipitation. The silver reineckate was removed by filtration, and the filtrate was treated with barium chloride solution to complete precipitation. After removal of the barium sulfate by centrifugation, the supernatant solution was evaporated to dryness under reduced pressure to yield 3.5 Gm. of quaternary chloride fraction C. Studies of this fraction will be reported at a later date.

Isolation of Alkaloids.—*Fraction A.*—The crude alkaloid fraction (13.5 Gm.) was dissolved in chloroform, and the chloroform solution was extracted with 3% sodium hydroxide solution. The aqueous alkaline layer was acidified with 6% hydrochloric acid, made alkaline with ammonia, and extracted with ether. The ethereal solution yielded 238 mg. of residue which resisted all attempts at crystallization. The chloroform solution was extracted with 6% hydrochloric acid and the acid extract was made alkaline and extracted with ether. Evaporation of the ether yielded 12.15 Gm. of residue. Crystallization from ether afforded 1.81 Gm. of crystalline product. Recrystallization from chloroform-methanol yielded colorless needles, m. p. 257–258°, $[\alpha]_D^{28}$ ±0° (c 1.20 $CHCl_3$). The melting point was not depressed on admixture of an authentic sample of *d,l*-tetrandrine (7). The paper chromatographic behavior and solution infrared and ultraviolet spectra were identical with those of the authentic *d,l*-tetrandrine sample.

The ethereal mother liquor was evaporated to dryness and the residue (10.25 Gm.) was dissolved in benzene and chromatographed on Merck acid-washed alumina (205 Gm.). The column was developed by successive elution with benzene and with benzene-methanol mixtures containing increasing proportions of methanol. The residue obtained from the benzene-eluted fractions was crystallized from chloroform-methanol to yield 1.31 Gm. of *d*-tetrandrine, m. p. 217–218°, $[\alpha]_D^{28}$ + 263° (c 0.80 $CHCl_3$). The identity of the material was established by mixed melting point, paper chromatographic, and

[1] The authors thank Rajaranga and Co., Madras, India, for gathering and fowarding the dried plant materials to us, and Dr. C. B. Sulochana, University Botany Laboratory, Madras, for confirming the identity of the plant.

infrared spectral comparisons with an authentic sample of *d*-tetrandrine (7).

The benzene-methanol and methanol fractions from the column yielded intractable amorphous mixtures.

Fraction B.—Crystallization of the crude alkaloid fraction (34.0 Gm.) from ether yielded 2.50 Gm. of crystalline first crop (B-1) and 5.10 Gm. of crystalline second crop (B-2). The mother liquor was evaporated to dryness (B-3).

The first crop solid (B-1) was extracted with benzene; 941 mg. remained insoluble. The benzene solution was evaporated to dryness and the residue was crystallized from chloroform-methanol to yield 292 mg. of *d*-tetrandrine, m. p. 217–218°. The benzene-insoluble solid was dissolved in chloroform and chromatographed on acid-washed alumina (20 Gm.). After development with additional chloroform, two distinct bands were evident; a brown band near the top of the column and a yellow band of higher R_f. Extrusion of the alumina, followed by separation of the bands by cutting permitted separation of the two zones. The two zones were eluted separately with methanol. The methanol solution corresponding to the brown zone yielded upon concentration 133 mg. of *d*-isochondrodendrine, m. p. 309–310°; $[\alpha]_D^{25} + 54°$ (c 0.80 pyr.). The melting point was not depressed on admixture with an authentic sample of *d*-isochondrodendrine (15). The paper chromatographic behavior and infrared spectrum (Nujol mull) were identical with those of the authentic *d*-isochondrodendrine sample. The methanol eluate corresponding to the yellow zone yielded 105 mg. of *d*-tetrandrine, m. p. 217–218°.

The second crop solid (B-2) was crystallized from chloroform-methanol to yield 2.68 Gm. of *d,l*-tetrandrine, m. p. 255–257°.

The mother liquor residue (B-3, 26.28 Gm.) was crystallized from acetone to yield 1.034 Gm. of *d*-tetrandrine, m. p. 216–218°. The acetone mother liquor was evaporated to dryness, and the solid residue was extracted with benzene. The benzene-insoluble solid was crystallized from acetone to yield 2.642 Gm. of solid, m. p. 158–167°. Recrystallization from pyridine-methanol gave 2.067 Gm. of crystalline material, m. p. 121–122°; $[\alpha]_D^{25} + 213°$ (c 0.90 $CHCl_3$). Recrystallization of a sample from methanol gave prisms, m. p. 177–179°. Recrystallization of a sample of the 121–122° melting material from acetone, seeding with a sample of high-melting fangchinoline (m. p. 237–238°) described earlier (7) led to the separation of crystalline product with m. p. 238–240°. The melting point was not depressed on admixture with the authentic sample of fangchinoline. The paper chromatographic behavior and infrared spectrum in chloroform were identical with those of the fangchinoline sample.

The mother liquors remaining from all the above portions of fraction A and fraction B were combined and purified by extraction with 6% hydrochloric acid, reprecipitation with ammonium hydroxide, and extraction with ether. The residue from the ethereal extract yielded to fractional crystallization from acetone, whereby 2.529 Gm. of *d*-tetrandrine and 1.699 Gm. of fangchinoline were obtained.

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Effects of Drugs on Conditioning in the Rat II

Synthesis of a Centrally Active Drug and the Effects of Nine Drugs on Operant Conditioning and Extinction

By M. D. ACETO†, V. D. LYNCH‡, and R. K. THOMS

In this study, an operant conditioning and extinction technique is used to compare the effects of various so-called tranquilizing agents. In addition, a method is presented for the preparation of 2-ethyl-2-(3-methyl)-butylmalondiamide. The statistical analysis of the data obtained is supported by observations which indicate that some of the effects of these drugs can be ascribed to secondary or side-effects.

THE TRANQUILIZING drugs comprise one of the areas of recent interest in the field of pharmacology. A considerable amount of experimentation and speculation has been undertaken in order to attempt to discover new compounds of this type and also to provide a basis for the understanding of the mode of action of these drugs.

However, little progress has been reported towards developing a unique test to determine the value of these compounds as regards their ability to alter behavior. The reason may be that tranquilizing drugs, like most other drugs, have potential, actual, primary, and/or secondary effects manifested as ataxia, depression, anorexia, ptosis of the eyelids, flaccid paralysis, and the like. These latter points constitute an important aspect in the development of tranquilizers and the effects of these drugs on animal behavior. Equally important is the aspect of chemical structure-activity relationships. Indeed, a large number of tranquilizers used therapeutically were the result of investigation in this area. With these effects in mind, this problem was designed and undertaken in an effort to contribute further to this area.

It was felt that one approach in determining whether or not the actual, potential, primary, and/or secondary effects of tranquilizers and other centrally active drugs contributed to overall behavior was to compare the same drugs at the same dose levels using two different experimental techniques. Specifically, it was felt that the results obtained by the application of operant conditioning techniques would not only provide information based on its own merits but would contrast or complement the results obtained by using an avoidance-escape technique (1). The two techniques are based entirely on different psychological principles and might serve to emphasize to what extent these actual, potential, primary, and/or secondary effects influence behavior and perhaps, within the limits of these two testing procedures, the type behavior.

Pfeiffer, *et al.* (2), in a comparison of the effects of meprobamate with other classes of drugs active in the central nervous system concluded that this compound has a prolonged barbiturate-like action mixed with a moderate degree of trimethadione effect on the brain. Furthermore, they claimed that the pharmacological action resembled phenobarbital or trimethadione even more than it did its congener mephenesin. They felt that meprobamate is not an ataractic drug as is chlorpromazine, and that it exhibits few properties of mephenesin.

It is interesting to note that Titus and Weiss (3) in an investigation of the metabolism of phenobarbital 2-C^{14} identified by means of paper chromatography nine metabolites, one of which was reported to be urea-C^{14} representing 2.5 per cent of the products. This metabolite strongly suggested that a certain amount of ring cleavage takes place in the metabolism of phenobarbital and possibly other barbiturates. An examination of the chemical structure of meprobamate, 2-methyl-2-*n*-propyl-1,3-propanediol dicarbamate, showed various features in common with the barbiturates. The most obvious is the quaternary carbon atom. Both hydrogens attached to the carbon atom at position 5 in barbituric acid must be replaced by alkyl or aryl groups in order to manifest hypnotic properties. If the carbonyl group at position 2 in a barbiturate such as amobarbital (5-ethyl-5-isoamylbarbituric acid) is removed, the result will be amides not unlike the carbamide groups of meprobamate. Many compounds with quaternary carbon atoms have been shown to possess

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interesting central effects. Already mentioned were the sedative and hypnotic effects of the barbiturates. Meprobamate shows tranquilizing and spasmolytic effects. Berger and Ludwig (4) examined various substituted propane-1,3-diols and found that diethyl propanediol (2,2-diethylpropane-1,3-diol) has the same effect on the spinal cord as mephenesin.

In view of the information available, it was proposed that various compounds closely related to both the barbiturates and meprobamate be investigated. Chosen for this study were 2-ethyl-2-(3-methyl)-butylmalondiamide and 2-ethyl-2-(3-methyl)-butylmalonic acid diethylester. The ester contains no nitrogen and it was thought that its effects might provide an interesting contrast with 2-ethyl-2-(3-methyl)-butylmalondiamide when tested on animal behavior.

Another compound, ethyl-2,4-toluene dicarbamate, was chosen for investigation because of the structural similarity between it and some of the *o*-toloxy derivatives investigated by Berger (5) and also because it contained the amide group which Toman (6) postulated should be present in order for a compound to have central depressant properties. Preliminary studies of several homologs of this compound demonstrated that this series of compounds possessed sedative and depressant properties worthy of further investigation.

EXPERIMENTAL

Preparation of 2-Ethyl-2-(3-methyl)-butylmalondiamide.—Eighteen grams of 2-ethyl-2-(3-methyl)-butylmalonic acid diethylester was hydrolyzed by refluxing on a steam bath for twenty-four hours with 30 ml. of a 50% hydroalcoholic (1:1) potassium hydroxide solution. The reaction mixture was diluted with 50 ml. of water and acidified to Congo red paper with concentrated hydrochloric acid. After cooling, the immiscible layers were separated and the aqueous portion was extracted with ether. The organic portions were combined and the ethereal solution was extracted with 5% aqueous sodium bicarbonate solution. The aqueous alkaline extract was acidified with hydrochloric acid and re-extracted with ether. Evaporation of the combined ether extracts yielded the free acid. After adding thionyl chloride dropwise to remove traces of water, an excess of the reagent was added, and the mixture was refluxed on a steam bath for one hour. The residual thionyl chloride was removed by distillation and the remaining acyl chloride was dissolved in 100 ml. of dry hexane. Dry ammonia was bubbled through the solution until the resulting suspension of diamide became a thick slurry. The solid was collected on a sintered-glass funnel and was washed with dry hexane, the filtrate being further treated with ammonia until no more precipitate formed. After being air dried, the crude diamide was extracted with hot ethanol and the filtered alcoholic solution was evaporated to dryness on a steam bath. After washing the residue with water to remove ammonium chloride and other impurities, it was dried and recrystallized from ethanol. The yield was 42.1%, m. p. 190–191° (uncorr.).

Anal.[1]—Calcd. for $C_{10}H_{20}N_2O_2$: N, 13.98; C, 59.97; H, 10.07. Found: N, 13.22, C, 59.62; H, 10.11.

Drugs.—The drugs and dosage levels are listed in Table I. Except for the diamide and dicarbamate (7) which were synthesized in our laboratories, all other drugs were obtained commercially or supplied by the manufacturer.[2] Distilled water was used as the solvent for the relatively water-soluble drugs. Methocel (0.5%) was used as the suspending agent for the poorly water-soluble drugs. All suspensions were shaken prior to use. In all cases, the drug was either dissolved or suspended so that the desired dose per Kg. of body weight of the rat was contained in a 1-ml. final volume of water. When not in use, the solutions were stored in a refrigerator.

Animals.—Descendants of a Wistar strain of rats were used in this study. As regards sex, the animals were chosen at random. These animals weighed at least 150 Gm. at the beginning of the test period. However, because of weight gains during the test period, some of the same animals weighed up to 310 Gm. at the end of the period. Each animal was housed separately, with a food intake maintained at between 8–11 Gm. daily. Water was allowed *ad libitum*.

Operant Conditioning and Extinction Procedure.—A bar-press apparatus was used for this procedure. This device permitted a food pellet to be dispensed each time the foot pedal inside the experimental chamber was depressed. Separate counters registered both the total number of bar presses and tablets dispensed. The food dispenser was arranged so that it could be disconnected during the extinction procedure. The experimental chamber was located in a windowless room adjacent to which was the room housing the control panel and recording devices.

After determining the operant level for each rat, it was conditioned to press the bar to obtain food. All the animals were conditioned to respond at least 100 times during a fifteen-minute session. With the pellet dispenser disconnected, each animal was then extinguished to a rate of ten bar presses or less during a fifteen-minute session. When a sufficient number of animals had been conditioned and extinguished, they were assigned to groups of four animals each. Each group was then assigned at random to one of the drugs and one of the dose levels being investigated.

Drugs were administered intraperitoneally in all cases, in order to insure optimum absorption. With the exception of reserpine and meprobamate, animals were injected with drugs one hour before admission to the test apparatus. Reserpine was administered two hours before and meprobamate

[1] The authors wish to thank Professor W. Williams who performed the nitrogen determination and Dr. Robert Gerraughty who performed the carbon and hydrogen determinations.

[2] The following drugs were supplied gratis from the firms listed: 2-ethyl-2-(3-methyl)-butylmalonic acid diethylester from Eli Lilly & Co.; meprobamate from Wyeth Laboratories; Thorazine hydrochloride from Smith Kline and French Laboratories; Atarax hydrochloride from Charles Pfizer and Co.; and Serpasil phosphate lyophilized crystals from Ciba Pharmaceutical Products, Inc.

TABLE I.—DRUGS AND DOSES USED IN OPERANT CONDITIONING TECHNIQUE[a]

Drug	Lot and Control No.	Low, mg./Kg.	Medium, mg./Kg.	High, mg./Kg.
Reserpine phosphate	Ciba E 4961	0.5	1.0	2.0
Chlorpromazine hydrochloride	SKF Cu 7664	0.5	2.0	4.0
Meprobamate	Wyeth L 3286-1	10.0	50.0	100.0
Hydroxyzine hydrochloride	Pfizer 71407	5.0	25.0	35.0
d-Amphetamine sulfate	Lack DA 675	0.1	1.0	5.0
Amobarbital	Eli Lilly AX-9643-F	5.0	10.0	20.0
Ethyl-2,4-toluene dicarbamate	...	50.0	75.0	90.0
2-Ethyl-2-(3-methyl)-butylmalondiamide	...	10.0	50.0	100.0
2-Ethyl-2-(3-methyl)-butylmalonic acid diethylester	Eli Lilly 11039	50.0	100.0	200.0
Control				

[a] All drugs given by i. p. injection.

one-half hour before the animal was placed in the conditioning chamber.

The test animals were reconditioned under the influence of drugs for two days. After the drug was stopped, the animal continued to recondition until a rate of at least 100 bar presses was achieved during a fifteen-minute session. The animal was then given the same drug and extinguished for two days. After the drug was withdrawn, extinction was continued until a rate of 10 bar presses or less was achieved in a fifteen-minute session. In those cases where extinction at the desired rate resulted on the first and/or second day in rats under the influence of a drug, at least one additional day was allowed for the animal to extinguish to the desired rate. It was felt this would disclose any obviously drug-induced depression of the extinction rate.

All animals were reconditioned and extinguished with drugs as many times as was feasible. A control group of four rats was used for each progressive reconditioning and extinction series.

RESULTS

The data obtained was analyzed statistically by means of the t test (8). Before subjecting the data to the t test, it was first determined that deviations from the means of the responses in both acquisition and extinction were normally distributed. Then, in order to compensate for the different learning abilities of the animals used, the total number of bar presses for each animal obtained during its first fifteen-minute session either under the influence of the drug or when serving as a control animal during acquisition (D) was subtracted from the total number of bar presses obtained during the first five 15-minute trials during which the animal first received reinforcement in response to each bar press (N). Negative numbers which resulted were then eliminated by adding to each result a constant (C), the largest negative number obtained from all the results. These numbers were used in the calculation of the t tests. The data obtained during extinction was similarly treated, with the exception that the total number of bar presses for each animal on the first 15-minute extinction trial was used compared to the total number of bar presses obtained during the first five 15-minute sessions of acquisition. Tables II and III list the results of the t tests for the drugs and dose levels shown in Table I during acquisition and extinction, respectively. Tables IV and V present the original and transformed data used in the calculation of the t tests for the different drugs which showed significant effects at the 0.05 level of significance when compared to the controls.

It should be noted that the measures $N - D$ are inversely related to the energizing effects of the drugs. Thus, if drugged animals pressed the bar significantly more than the controls, the sums in the columns labeled $C + N - D$ for the drugged animals would be less than the sums in the column labeled $C + N - D$ for the control.

TABLE II.—TABLE OF t TESTS DERIVED FROM THE COMPARISON OF RESPONSES MADE BY DRUGGED ANIMALS AND CONTROL ANIMALS DURING ACQUISITION OF OPERANT CONDITIONING[a]

Drug	Dosage Level		
	Low	Medium	High
Reserpine PO_4	1.68	0.40	0.31
Chlorpromazine HCl	1.56	1.09	1.67
Meprobamate	0.67	0.08	3.44
Hydroxyzine HCl	1.18	1.11	1.33
d-Amphetamine SO_4	0.42	2.29	0.43
Amobarbital	3.19	0.78	2.14
Ethyl-2,4-toluene dicarbamate	0.14	0.44	0.38
2-Ethyl-2-(3-methyl)-butylmalondiamide	0.82	0.03	0.66
2-Ethyl-2-(3-methyl)-butylmalonic acid diethylester	4.03	0.28	0.31

[a] Value of t at the 0.05 level of significance for 6 degrees of freedom is 2.447 (8).

TABLE III.—TABLE OF t TESTS DERIVED FROM THE COMPARISON OF RESPONSES MADE BY DRUGGED AND CONTROL ANIMALS DURING OPERANT EXTINCTION[a]

Drug	Dosage Level		
	Low	Medium	High
Reserpine PO_4	1.10	1.05	2.37
Chlorpromazine HCl	1.87	1.99	1.10
Meprobamate	0.65	2.21	1.55
Hydroxyzine HCl	1.56	2.37	1.13
d-Amphetamine SO_4	0.29	3.10	3.29
Amobarbital	1.17	0.18	0.53
Ethyl-2,4-toluene dicarbamate	0.01	2.03	0.21
2-Ethyl-2-(3-methyl)-butylmalondiamide	2.14	1.09	0.59
2-Ethyl-2-(3-methyl)-butylmalonic acid diethylester	1.42	1.33	2.17

[a] Value of t at the 0.05 level of significance for 6 degrees of freedom is 2.447 (8).

TABLE IV.—ORIGINAL AND TRANSFORMED DATA OF THOSE DRUGS SHOWING SIGNIFICANT EFFECTS ON OPERANT CONDITIONING AT THE 0.05 LEVEL OF SIGNIFICANCE WHEN COMPARED TO CONTROLS

Group No.	Rat No.	N	D	N − D	C + N − D	C + N − D 3rd Control
		Amytal, 5 mg./Kg. (Third Reconditioning)				
11	Y-27	4	115	−111	29	116
	Y-29	59	71	− 12	128	154
	Y-31	4	91	− 87	53	159
	Y-33	12	136	−124	16	253
		Meprobamate, 100 mg./Kg. (First Reconditioning)				
11	Y-27	4	48	− 44	96	145[a]
	Y-29	59	61	− 2	138	224[a]
	Y-31	4	82	− 78	62	167[a]
	Y-33	12	123	−101	39	253[a]
		2-Ethyl-2-(3-methyl)-butylmalonic Acid Diethylester, 50 mg./Kg. (First Reconditioning)				
13	Y-21	62	84	− 22	118	145[a]
	Y-23	4	88	− 84	52	224[a]
	Y-35	7	58	− 51	89	167[a]
	Y-40	187	105	82	58	256[a]

[a] First control.

TABLE V.—ORIGINAL AND TRANSFORMED DATA OF THOSE DRUGS SHOWING SIGNIFICANT EFFECTS ON OPERANT EXTINCTION AT THE 0.05 LEVEL OF SIGNIFICANCE WHEN COMPARED TO CONTROLS

Group No.	Rat No.	N	D	N − D	C + N − D	C + N − D 2nd Control
		Dextro-Amphetamine Sulfate, 1 mg./Kg. (Second Extinction)				
3	Y-35	79	55	15	84	131
	Y-37	55	51	− 4	65	118
	2NY-17	69	87	− 18	51	61
	Y-28	19	6	13	82	126
		Dextro-Amphetamine Sulfate, 5 mg./Kg. (Third Extinction)				
11	Y-27	128	0	128	197	71[a]
	Y-29	133	0	133	202	101[a]
	Y-31	114	0	114	183	122[a]
	Y-33	285	0	285	354	93[a]

[a] Third control.

SUMMARY AND CONCLUSIONS

Little has been reported previously concerning the acquisition of bar-pressing habit as a measure of the effects of drugs. As a rule, most of the experimentation of this nature has been concerned with the effects of drugs on already conditioned operant behavior, or on the extinction of such behavior. In the research presented here, it was proposed originally that an attempt would be made to determine the effects of drugs on the acquisition and extinction of operant behavior with the possibility in mind of comparing this technique to avoidance-escape conditioning relative to the effects of drugs. It was determined, however, that the experimental design which was followed would allow only a gross measure of the effect of drugs on operant conditioning and extinction. This precluded any comparative evaluation of the two measures.

The results of the *t* tests in both the operant conditioning and extinction procedures suggested statistically subliminal effects which probably were not detected because of the "noise" in the experimental design.

The results obtained through these procedures do allow some pertinent conclusions to be drawn, however. Thus, the effects of *d*-amphetamine sulfate indicated that 1 mg./Kg. appears to increase bar-press activity and 5 mg./Kg. appears to decrease bar-press activity when compared to the controls. An examination of the first day's effects of the different drugs tested at the dose ranges shown in Table I on both conditioning and extinction indicated that only those drugs which interfered with normal motor activity (e. g., amobarbital, meprobamate, and 2-ethyl-2-(3-methyl)-butylmalonic acid diethylester) and those drugs with anorexic properties (e. g., dextro-amphetamine sulfate) had significant effects when compared to the controls. While the *t* tests demonstrate that these several drugs do affect behavior significantly, these results cannot be construed to mean that those drugs had any significant effect on the contingent learning process because of the associated effects of these compounds. On the other hand, it would be erroneous to suggest that these drugs had no effect on psycho-

logical processes. Rather it is suggested that a refinement of technique is required to differentiate between the various effects.

In the course of these experiments, it was further noted that the drugs used in this study had more pronounced pharmacological effects on the fasted animals as compared to the animals used in avoidance-escape training which were allowed to feed *ad libitum*.

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In Vitro Tests for Measuring Antibacterial Activity of Toilet Soap and Detergent Bars

By L. J. VINSON, E. L. AMBYE, A. G. BENNETT, W. C. SCHNEIDER, and J. J. TRAVERS

Two new simple *in vitro* screening tests are described which have proved valuable in predicting the degerming effectiveness of germicidal toilet bars under regular use conditions. The tests, skin disk substantivity and finger imprint tests, measure the residual antibacterial activity of skin treated with soap solutions followed by a water rinse. Thus, a soap germicide is evaluated on the basis of its capacity to become adsorbed to skin and its ability, at low concentrations, to inhibit the growth of bacteria. *In vitro* test data are described on 3,4′,5-tribromosalicylanilide and other germicides incorporated into soap and detergent bars. Good correlation between the new *in vitro* test results and regular hand washing results (serial basin wash test) is achieved.

IN RECENT YEARS germicides are being employed more and more in toilet bars, contributing important properties not possessed by plain soaps. Although antiseptic agents have been used in soaps for many years it was only with the introduction of soap germicides like the bisphenolics (1, 2), tetramethylthiuramdisulfide (3, 4), and trichlorocarbanilide (5) that a substantial degerming of skin was achieved in regular washing. These germicides all possessed in common two properties to varying degrees: (*a*) antibacterial action at low concentrations against Gram-positive bacteria (micrococci), normal residents of skin which are not destroyed by exposure to plain soap washing; (*b*) an affinity for skin such that during washing with a soap, the germicide is adsorbed on the epidermis and is not washed out during the rinse operation. The presence of trace amounts of an effective germicide on the skin has the effect of suppressing the growth of the resident bacteria that feed on skin detritus. Thus, the typical "body odor" which arises from bacterial growth can be reduced.

Standard bacteriological procedures like the phenol coefficient test or toxic dilution tests, employing seeded agar plates or broth tubes, are not good methods for predicting the effectiveness of germicides in toilet bars. A preparation may be effective at very low concentrations as demonstrated in such tests but may not be substantive to skin, or it may prove incompatible with the vehicle. The practical usefulness of germicidal soaps is determined in the serial basin wash test which provides antibacterial data in terms of per cent reduction in hand bacterial counts after regular use of the test bar. This method, however, is tedious and time consuming and care must be taken in interpreting results with small panels (6). Another practical test for assessing germicidal bars is to determine the inhibitory effect on perspiration odor development of washed axillae (3).

In the screening of a new germicide simple tests are necessary to determine not only the minimum effective concentration in various

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vehicles, but also to evaluate its skin substantivity. It is the purpose of this report to describe three *in vitro* tests which are used routinely in our laboratory in predicting the practical activity of germicidal soaps in regular use.

METHODS

Soap germicides were evaluated in three types of toilet bars: soap, soap containing a synthetic detergent, and a synthetic detergent bar, all employed as 8% solutions, a concentration close to that found in lather. The three tests employed are: (*a*) filter paper disk test, (*b*) skin disk substantivity test, and (*c*) the finger imprint test. The soap germicides evaluated in these tests were: 4′,5-dibromosalicylanilide (DBS); 3,4′,5-tribromosalicylanilide (TBS); 3,4,4′ - trichlorocarbanilide (TCC); 2,2′ - methylenebis(3,5,6-trichlorophenol) (G-11); and tetramethylthiuramdisulfide (TMTD).

Filter Paper Disk Test.—This test is a preliminary screening method used for determining whether a germicide shows activity in a soap solution. Standard Penassay disks[1] are placed on nutrient agar seeded with the test organism, *Staphylococcus aureus*, and wetted with 0.05-ml. aliquots of the germicidal test solution. The plates are incubated at 37° overnight and examined for zones of inhibition. The minimum effective level of the antibacterial agent in soap to produce a zone of inhibition is readily determined. It is important to note that the size of zone developed is not necessarily an indication of the practical potency of the germicidal soap. Also, findings obtained in the filter paper disk test do not provide information on the behavior of the soap germicide on skin.

Skin Disk Substantivity Test.—This test measures the antibacterial activity remaining on skin disks after immersion in a germicidal soap solution and rinsing in water. Special calf skin disks,[2] 8 mm. in diameter, are employed. The disks are steeped in the germicidal soap solutions maintained in a water bath at 45°. They are removed, rinsed thoroughly in running water, and then placed on agar seeded with *Staphylococcus aureus*.

The development of zones of inhibition indicate that the adsorbed germicide migrates into the agar and effectively inhibits the growth of the test organism. The size of the inhibition zones is a function not only of the potency of the germicide but also of the diffusion rate of the germicide molecule. Consequently, a greater zone of inhibition for one germicide in this test does not always signify that it would be superior in a practical way to a second germicide giving a smaller zone.

Conditions were varied in order to provide antibacterial data that would predict activity under actual use conditions. In one study, the time of immersion of the skin disks in the germicidal soap solution was varied. The disks were rinsed and allowed to remain on the agar during an incubation period of eighteen to twenty-four hours. Data cited in Table I indicate that germicide pickup by the skin disks increases as the immersion time is increased, up to sixty minutes.

Table I.—Effect of Immersion Time of Skin Disks in Two Germicidal Soap Solutions on Antibacterial Activity

Immersion Time, Min.	Zones of Inhibition, mm. for Skin Disks Immersed in 8% Soap-Detergent Solutions Containing Germicides	
	2% TCC[a]	0.5% TBS[a]
1	1.5	2.9
1	2.1	3.9
2	2.4	3.6
5	2.3	5.6
10	2.3	7.2
15	1.9	6.6
30	1.3	8.6
60	5.2	12.8

[a] Basis product weight.

Table II.—Reproducibility of Skin Disk Substantivity Results on Six Production Samples of a Soap-Detergent Bar Containing 0.5% TBS

Sample Nos.	1	2	3	4	5	6
Ratings[a]	4	4	2	4	4	4
	4	4	4	2	4	4
	4	2	3	3	4	2
	4	4	3	3	4	3
	4	4	4	4	3	3
	4	3	4	3	1	4
Av. Rating	4	3.5	3.3	3.2	3.3	3.3

[a] Six replicates per sample.

In another study, the contact time on seeded agar of the treated disks (immersed in soap solution for fifteen minutes) was investigated. Instead of allowing the disks to remain in contact with the agar overnight, the disks were removed at different time intervals from three to sixty minutes after agar contact. The plates with the disks removed were then incubated overnight and the contact zones examined for varying degrees of clarity as a measure of growth inhibition. Ratings were then assigned to the contact zones on the basis of varying degrees of growth as follows: rating of 4, sharply outlined clear area free of growth (excellent activity); rating of 3, clear area of no growth with hazy periphery (good activity); rating of 2, area showing partial growth (fair activity); rating of 1, area showing growth almost equal to surrounding agar (slight activity); and rating of 0, area showing growth equal to surrounding agar (no activity).

Results of this study indicated that soaps containing effective germicides like TBS, G-11, or TMTD impart activity to skin disks, which becomes evident after only fifteen to thirty minutes contact time with seeded agar plates. This relatively short

[1] Standard filter paper disks, 12.7 mm. diam. (No. 740-E) by Carl Schleicher and Schuell Co., Keene, N. H., are employed in the assay of penicillin and other antibacterial substances.

[2] Prepared from pickled calf skin which is processed as follows: The skin is depickled overnight in four times its weight of an aqueous solution of 3.12% sodium chloride and 0.25% sodium bicarbonate. When the skin reaches a pH of 5–6, as determined on liquid squeezed therefrom, it is rinsed thoroughly in water to remove the salt completely. It is then dehydrated by placing in 95% alcohol (two daily changes) and finally in acetone for several days. The skin piece is drained and dried, grain side down, by securing to a wooden board in a well-ventilated room. Disks can be punched out from the skin which is soft and supple. The disks are readily hydrated by steeping in water for at least fifteen minutes prior to use in the tests.

We are indebted to Dr. D. E. Kvalnes and Dr. Paul Lipsitz, DuPont's Jackson Laboratories, for providing us with information on preparing dehydrated calf skin.

TABLE III.—SKIN SUBSTANTIVITY TEST DATA ON SIX SOAPS CONTAINING ANTIBACTERIAL AGENTS OF VARYING ACTIVITY

	Soap A	Soap B	Soap C	Soap D	Soap E	Soap F
	4	1	0	0	3	4
	4	1	0	0	1	4
	4	2	0	0	3	4
	3	2	1	0	2	4
	4	2	1	0	3	4
	2	2	1	0	2	4
Av. Rating	3.5	1.7	0.5	0	2.3	4
Type of Activity	Good to excellent	Poor	Very poor	No activity	Fair	Excellent

period of time for migration of the antibacterial agent from the disk into the agar in amounts sufficient to inhibit the test organism is considered an important property of a soap germicide. It is not enough that a germicide be adsorbed on skin; it also should be reasonably free to migrate, since bacteria exist in depth in the epidermis.

The skin disk substantivity test that has proved to be a reliable screening test in the selection of soap germicides is as follows: replicates of six disks are employed in the testing of each germicidal soap solution. The disks are steeped for fifteen minutes in 8% test soap solutions maintained at 45° followed by a thorough rinse in running water for an additional fifteen minutes. The disks are pressed free of excess water and placed on nutrient agar seeded with *Staphylococcus aureus*[3] or other test organisms for a period of thirty minutes. They are then removed and the plates incubated overnight. The contact zones are examined for growth inhibition and rated according to scores given in the foregoing.

Typical results are given in Table II for six different production samples of a soap-detergent bar containing 0.5% TBS. Reproducibility and precision of results were good and in every instance the average rating was over 3, indicating good to excellent antibacterial activity. In Table III data are given for six soaps from no apparent antibacterial activity to excellent activity (ratings from 0 to 4).

Finger Imprint Test.—The finger imprint test is another useful screening test employing human subjects. It is routinely used to determine the skin-substantive properties of germicidal soaps under simulated wash conditions. Hands are prewashed for one minute with plain soap. The individual fingers of each subject are then immersed simultaneously for thirty seconds into several 8% test solutions maintained at 45° (thumbs are usually not used). The fingers are thoroughly rinsed in running water for one minute, dried, and then placed lightly for thirty seconds on agar seeded with *Staphylococcus aureus*.[4] The selection of the thirty-second period for finger immersion in the test solutions as well as for contact with the seeded agar was made on the basis of practical considerations and the fact that the findings correlated well with serial basin wash results.

The plates are incubated at 37° overnight and the contact areas examined for growth inhibition.

[3] A 1% inoculum of a twenty-four-hour growth culture of *S. aureus* (FDA No. 209) in nutrient agar. This melted agar (11 ml.) is poured onto a hardened based layer of sterile nutrient agar (50 ml.) contained in a large Petri dish (150 mm. diam.). The disks are placed on the agar with the smooth side down.

[4] A thin layer (11 ml., 150-mm. diameter Petri dish) of hardened nutrient agar containing a twenty-four-hour growth culture of *S. aureus* at a 1% level.

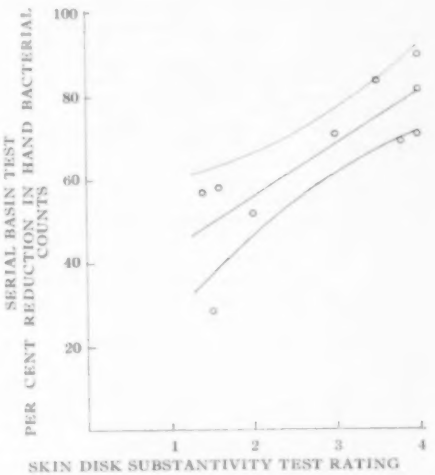

Fig. 1.—Regression of per cent reduction in hand bacterial counts on skin disk substantivity test rating, and 95 per cent confidence interval for the line; $r = 0.83$; $Y = 28 + 13.2X$.

Ratings of the test areas are the same as described for the skin disk substantivity test. A variation of the finger imprint test that has been employed is to lather one hand with a test soap for thirty seconds followed by a thorough rinse. The other hand is similarly washed with a second bar. Thus, comparisons of hands washed with two soaps can be made on the same plate.

RESULTS AND DISCUSSION

In vitro data employing the three tests described in the foregoing are presented in Table IV. The results are compared with regular hand washing data obtained for the test bars in a standard serial basin wash test (6). Bars showing high ratings, around 3 or higher, in the skin disk substantivity test and/or finger imprint test generally give very satisfactory degerming activity in the serial basin wash test. One exception appears to be TCC which yields only fair finger imprint activity but shows reasonably good reduction in hand bacterial count. For example, at a 2.0% level in a soap bar containing a dispersant agent, TCC yields ratings of 1.6 and 0.9 in the skin disk substantivity and finger imprint tests but still results in fairly good reduction (58%) in hand bacterial count.

TABLE IV.—*IN VITRO* AND SERIAL BASIN WASH DATA ON TOILET BARS CONTAINING TBS AND OTHER GERMICIDES

Vehicle	Per Cent Germicide	*In Vitro* Test Data: Filter Paper Disk, No. of Disks	Filter Paper Disk, Av. Zone, mm.	Skin Disk Substantivity, No. of Disks	Skin Disk Substantivity, Av. Rating	Finger Imprint, No. of Subjects	Finger Imprint, Av. Rating	Reduction in Hand Bacterial Counts, Serial Basin Wash Test, No. of Subjects	Reduction in Hand Bacterial Counts, Serial Basin Wash Test, % Reduction
Soap	1% TCC	8	2.2	6	3.0	66	1.5	18	71
Soap	1% TMTD	28	10.8	12	4.0	52	2.3	10	82
Soap	0.5% TBS	21	1.8	26	3.8	219	3.0	26	69
Soap	0.5% TCC + 0.5% G-11	22	3.6	12	4.0	63	3.6	9	71
Soap-detergent bar	0.5% TBS	42	1.0	12	3.5	162	2.7	32	84
Soap-detergent bar	2.0% TCC	20	3.5	27	1.6	70	0.9	17	58
Soap-detergent bar	0.5% DBS	27	2.0	15	1.4	13	1.2	8	57
Detergent bar	0.5% TBS	16	2.0	9	1.5	37	0.9	7	29
Detergent bar	1.0% TBS	13	2.0	8	2.0	54	2.2	6	52
Detergent bar	1.0% TCC + 0.5% G-11	4	5.2	2	4.0	98	3.5	9	90

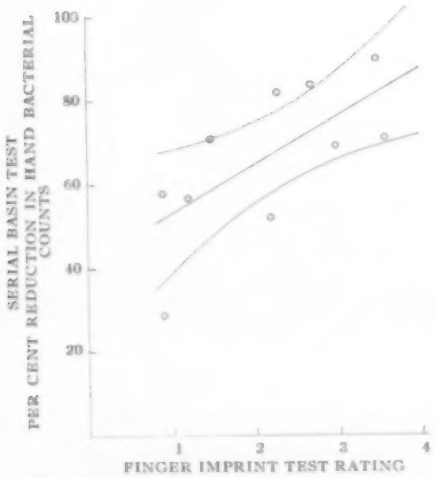

Fig. 2.—Regression of per cent reduction in hand bacterial counts on finger imprint test rating, and 95 per cent confidence interval for the line; $r = 0.69$; $Y = 40 + 12.2X$.

Correlation curves relating skin disk substantivity and finger imprint data to serial basin wash data show a high degree of significance with correlation coefficients of 0.83 and 0.69, respectively (Figs. 1 and 2). Correlation between filter paper disk data and hand washing results is not good. This is not surprising since the factor of skin substantivity is not considered.

It is interesting to note the effect of the vehicle on the degerming activity of TBS. At a 0.5% level in soap or soap containing a dispersant agent, TBS gives satisfactory *in vitro* and hand washing results. The activity becomes significantly less, however, in a detergent base. More detailed information on the relation of structure to activity of the halogenated salicylanilides is given in another report (7).

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Synthesis and Germicidal Activity of Halogenated Salicylanilides and Related Compounds

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Synthesis and germicidal activity in the presence of detergents are reported for halogenated derivatives of the following classes of compounds: salicylanilides, homologs of salicylanilide, salicylanilides with substituents other than halogen or alkyl groups, other amides of salicylic acid, anilides of other acids, and Schiff's bases of salicylaldehyde. The anilides were prepared by first halogenating the acid or aniline in the desired manner and then reacting these products to form the amide linkage. In some cases, when the position of the incoming halogen would be unambiguous, the anilides were further halogenated to form new products. Bactericidal effectiveness and skin substantivity of the products were evaluated using filter paper and skin disk substantivity tests in the presence of soap or detergent. High activity in soap was shown by salicylanilides halogenated in these positions: 4′,5; 2′,4′,5; 3,4′,5; 3′,4′,5; 4,4′,5; 2′,3,4′,5; 3,3′,4′,5; 2′,3,4′,5,5′. Certain homologs and nitro-derivatives also possessed high activity.

THE GERMICIDAL properties of the halogenated salicylanilides have received considerable attention (1–19). Few details are available, however, on the activity of these materials in the presence of soap and no data have been published on their substantivity to skin. Ninety-two halogenated salicylanilides and related compounds have now been synthesized and tested for germicidal activity and skin substantivity in the presence of soap or detergent. The details of synthesis and the results of germicidal evaluation in the filter paper disk and skin disk substantivity tests are presented here.

EXPERIMENTAL

The compounds prepared are listed in Table I. The general preparative procedures are illustrated with specific examples. Analytical data and literature references for individual compounds are shown in Table I.

1a. **3,4′,5-Tribromosalicylanilide.**—A slurry of 23.7 Gm. of 3,5-dibromosalicylic acid and 13.8 Gm. of 4-bromoaniline in 200 ml. of chlorobenzene was refluxed with 5.4 Gm. of phosphorus trichloride and 0.5 Gm. of aluminum chloride for four hours and then filtered hot. The filtrate deposited 28 Gm. (78%) of 3,4′,5-tribromosalicylanilide, melting at 222–226°.

1b. **2′,3,4′,5-Tetrabromosalicylanilide.**—A slurry of 10 Gm. of dibromosalicylic acid and 9 Gm. of 2,4-dibromoaniline in 200 ml. of chlorobenzene was refluxed with 1.5 ml. of phosphorus trichloride for three hours and then decanted hot. The cold solution deposited 12 Gm. (68%) of 2′,3,4′,5-tetrabromosalicylanilide, melting at 203–208°.

2. **5-Bromo-4′-chlorosalicylanilide.**—A slurry of 6 Gm. of 4′-chlorosalicylanilide (melting at 164–168°; Cl found, 13.93%) in 60 ml. of acetic acid was treated with 4 Gm. of bromine and then heated until fumes of hydrogen bromide appeared. The next day 7 Gm. (86%) of 5-bromo-4′-chlorosalicylanilide, melting at 238–243°, was collected.

3. **2-Acetoxy-4′,5-dibromobenzanilide.**—A solution of 18 Gm. of 4′,5-dibromosalicylanilide was prepared in 0.5 L. of water containing 6 Gm. of sodium hydroxide. Some suspended particles were filtered off and the solution treated with 6 Gm. of acetic anhydride. The solid was filtered and washed thoroughly with chloroform. The chloroform was separated, concentrated, and cooled, whereupon 5 Gm. of 2-acetoxy-4′,5-dibromobenzanilide, melting at 155–164°, was collected.

4. **4-Bromo-N(2-hydroxybenzylidene) Aniline.**—Ethanol solutions of 6 Gm. of salicylaldehyde, and 8.6 Gm. of 4-bromoaniline were prepared separately and mixed. The Schiff's base precipitated and was filtered. Recrystallization from alcohol gave 10 Gm. (72%) of yellow platelets melting at 110–112°.

5. **3,4′,5-Tribromo-2-aminobenzanilide.**—A solution of 2.65 Gm. of 2-aminobenzanilide in 100 ml. of acetic acid and 3.8 Gm. of triethylamine was treated with 6 Gm. of bromine. The next day the product was collected. After two recrystallizations from alcohol it weighed 1 Gm. (18%) and melted at 215–218°.

DISCUSSION

When salicylanilide or a pure substituted salicylanilide is reacted with bromine the compounds which result will depend on the relative reactivities of the unsubstituted sites in the salicylanilide molecule. If the reaction involves only one mole of bromine and if there is one site in the molecule much more reactive than any other, a relatively pure compound can be obtained.

A number of compounds listed in Table I were prepared by bromination of halogenated salicylanilides (see Experimental, method 2). Sixteen compounds synthesized in this manner are listed in Table II. The purity of these materials was estimated from the sharpness of the melting points (M. P.) and from the elemental analyses (Anal.); their identities were established by a comparison of their melting points and infrared spectra (IR) with those of known com-

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TABLE I.—SYNTHETIC MATERIALS RELATED TO SALICYLANILIDE

Substituents and Positions	Method of Prepn. and Purifn.[a,b]	Melting Point, °C. Found	Melting Point, °C. Literature	Analysis Elem.	Analysis Calcd.	Analysis Found	Lit. Refs.
Halogenated Salicylanilides[c]							
4′-Br	1a Pur Recr. P	173–175	...	Br	27.35	26.95	(5)
5-Br	1b Recr. A	225–227	222 (21)	Br	27.35	26.92	(5, 21)
2′,5-Br_2	1b Recr. P	189–191	...	Br	43.08	43.04	...
3,4′-Br_2	1b Recr. TA	214–216	...	Br	43.08	42.80[d]	...
3,5-Br_2	1a Recr. T	138–140	139–140 (22)	Br	43.08	42.98	(5, 22)
3′,5-Br_2	1a Recr. P	222–225	...	Br	43.08	42.76	...
4′,5-Br_2	1b Recr. APP	240–241	233–234 (8)	Br	43.08	43.25	(5–8, 13)
2′,3,5-Br_3	1b	178–179	...	Br	53.28	53.16	...
2′,4′,5-Br_3	1a Recr. PAP	233–236	...	Br	53.28	52.95[d]	(13)
3,3′,5-Br_3	1b Recr. P	155–158	...	Br	53.28	53.38	...
3,4′,5-Br_3	1b Recr. PAP	225–227	...	Br	53.28	52.91	...
3′,4′,5-Br_3	1b Recr. A	252–254	...	Br	53.28	52.82	...
4,4′,5-Br_3	1b Recr. AP	258–260	...	Br	53.28	52.89	...
2′,3,4′,5-Br_4	1b Recr. PP	206–208	...	Br	60.44	60.46	...
3,3′,4′,5-Br_4	2 Recr. P	192–194	...	Br	60.44	59.52	...
2′,3,4′,5,5′-Br_5	1b Recr. A	214–216	...	Br	65.74	65.28	...
2′-Cl	1b Recr. PA	167–168	168–169 (23)	Cl	14.32	14.15	(23)
4′-Cl	1a Recr. P	166–168	167–168 (8)	Cl	14.32	13.93	(5, 8, 23–25)
5-Cl	1a Recr. AP	209–211	203–204 (27) 207 (28)	Cl	14.32	14.41	(5, 15, 26–28)
2′,5-Cl_2	1b Recr. P	230–232	228 (29)	Cl	25.13	24.58	(5, 28, 29)
3′,4′-Cl_2	1b	213–216	...	Cl	25.13	25.35[d]	(9)
3,5-Cl_2	1a[e] Recr. P	133–135	134–135 (30) 134–135 (22)	Cl	24.13	24.50	(5, 19, 22, 30)
4′,5-Cl_2	1b Recr. A	231–232	215–216	Cl	25.13	24.58	(13, 15, 27)
2′,4′,5-Cl_3	1b	216–219	218–219 (11)	Cl	33.60	33.07	(2, 3, 5, 11, 13, 15)
3,3′,5-Cl_3	1b Recr. PA′P	135–150	...	Cl	33.60	33.11	...
3,4′,5-Cl_3	1b Recr. P	190–192	188–189	Cl	33.60	33.00	(5, 19, 30)
3′,4′,5-Cl_3[f]	1a Recr. PA	246–248	246–248	..	...	...	(1–3)
2′,3,4′,5-Cl_4	1b Recr. P	180–181	174–175 (27)	Cl	40.40	40.00	(5, 27)
3,3′,4′,5-Cl_4	1b Recr. B	149–151	162 (11)	Cl	40.40	40.22	(11, 17)
2′-Br-5-Cl	1b Recr. P	179–182	178 (8)	#	1.014	1.007	(5, 8)
4′-Br-5-Cl	1a Recr. PPA	224–226.5	222 (8)	#	1.014	0.995	(5–8, 13)
5-Br-2′-Cl	2 Recr. PA	202–203	...	#	1.014	1.008	(5)
5-Br-4′-Cl	2 Recr. P	238–243	241–244 (8)	#	1.014	1.005	(5–8)
2′,4′-Br_2-5-Cl	1b Recr. PA	225–226	...	#	1.280	1.251	(5, 13)
3,4′-Br_2-5-Cl	1a Recr. A	210–214	...	#	1.280	1.272	...
3,5-Br_2-2′-Cl	1b Recr. P	167–169	...	#	1.280	1.242[d]	(31)
3,5-Br_2-3′-Cl	1a Recr. AA	160–162	...	#	1.280	1.250	(31)
3,5-Br_2-4′-Cl	1a Recr. A	199–203	200 (8)	#	1.280	1.274	(6–8)
4′,5-Br_2-2′-Cl	1b Recr. P	236–241	...	#	1.280	1.284	...
4′,5-Br_2-3-Cl	1a Recr. P	220–226	...	#	1.280	1.276	...
3-Br-4′,5-Cl_2	1b Recr. P	187–190	(8)	*	1.314	1.301	(8)
4′-Br-3,5-Cl_2	1a Recr. P	209–212	...	#	1.314	1.288	...
5-Br-3,4′-Cl_2	1b Recr. A	195–200	...	#	1.314	1.312	...
5-Br-3′,4′-Cl_2	1b Recr. P	235–238	...	#	1.314	1.314	...
3,4′,5-Br_3-3′-Cl	2 Recr. A	178–181	...	#	1.459	1.449	...
3,5-Br_2-2′,4′-Cl_2	1b Recr. P	179–193[h]	178–179 (11)	#	1.505	1.505	(11, 17)
3,5-Br_2-2′,5′-Cl_2	1a Recr. P	156–159	...	#	1.505	1.475	...
3,5-Br_2-3′,4′-Cl_2	1b Recr. A	171–172.5	176–178 (11)	#	1.505	1.481	(11, 17)
4′-Br-3,3′,5-Cl_3	2[i] Recr. P	172–175	...	#	1.562	1.544	...
3,4′,5-Br_3-2′,5′-Cl_2	1b Recr. A	187–189	...	#	1.638	1.622	...
3,5-Br_2-4′-F	1b[j] Recr. AA	183–193	...	Br	41.08	40.59	...
Halogenated Sulfur Derivatives of Salicylanilide							
3,5-Br_2-2′-SO_2NH_2	1b Recr. A	228–231	...	Br	35.50	34.93	...
3,4′,5-Br_3-2′-SO_2NH_2	1b Recr. PP	249–251	...	Br	45.32	44.15	...
3,5-Br_2-4′-SO_2NH_2	1b	263 decompn.	...	Br	35.50	34.95	...
3,5-Br_2-2′-SH[k]	1b Recr. P	171–179	...	Br	39.75	42.05	...
Halogenated Homologs of Salicylanilide							
3,4′,5-Br_3-N-CH_3	2 Recr. P	178–180	...	Br	51.67	51.22	...
4′ Br-3-CH_3	1b Recr. P	150–154	...	Br	26.10	26.09	...
3′,5-Br_2-3-CH_3	1b Recr. PP	136–147	...	Br	41.51	41.64	...
4′,5-Br_2-3-CH_3	2 Recr. P	158–159	162–163 (34)	Br	41.51	41.41	(34)
3′,4′,5-Br_3-3-CH_3	2[l] Recr. PP	211–213	...	Br	51.67	50.72	...
5-Br-2′,4′-Cl_2-3-CH_3	1b	157–158	...	#	1.265	1.261	...
5-Br-2′,5′-Cl_2-3-CH_3	1b	169–173	...	#	1.265	1.255	...
3,5-Br_2-3′-CH_3	1b Recr. P	158–164	...	Br	41.51	41.78	...
3,4′,5-Br_3-3′-CH_3	2	165–169	...	Br	51.67	51.56	...
3,5-Br_2-4′-CH_3	1b	161–162 173–175	...	Br	41.51	41.73	...

TABLE I. (*continued*)

Substituents and Positions	Method of Prepn. and Purifn.[a,b]	Melting Point, °C. Found	Melting Point, °C. Literature	Analysis Elem.	Analysis Calcd.	Analysis Found	Lit. Refs.
5-Br-3′-Cl-4′-CH_3	1b Recr. PA	236–238	...	[g]	0.972	0.969	...
3,5-Br_2-3′-Cl-4′-CH_3	1b	185–188	...	[g]	1.237	1.233	...
5-Br-3,4′-$(CH_3)_2$	2	153–163	...	Br	24.96	25.84	...
Other Halogenated Salicylanilides							
5,5′-Cl_2-2′-OCH_3	1a Recr. PA	223–224	...	Cl	22.72	22.21	...
4′-Br 4-$NHCOCH_3$	1b Recr. P	258–265	...	Br	22.89	20.76	...
5-Br-3′,4′-Cl_2-3-C_6H_5	1b Recr. F	100–106	...	[g]	1.085	0.986	...
4′,5-Br_2-O-Acetyl	3	155–164		Br	38.69	38.41	
3′,4′-Cl_2-3-NO_2	1b Recr. P	173.8–174.2	...	Cl	21.68	21.16	...
3′,4′-Cl_2-5-NO_2	1b Recr. P	280–282 decompn.	...	Cl	21.68	20.95	...
5-Br-3′,4′-Cl_2-3-NO_2	1b Recr. P	192.5–194	...	[g]	1.169	1.083	...
4-Hydroxybenzanilides							
4′-Br	1a Recr. PCP	203–213	...	Br	26.09	24.65	...
3,4′-Br_2	2 Recr. P	203–211	...	Br	43.08	40.98	...
3,5-Br_2	1b Recr. P	202–218	...	Br	43.08	43.46	...
3,4′,5-Br_3	1a Recr. P	214–218	...	Br	53.28	53.01	...
Resorcylanilides							
4′-Br	1a Recr. PCC	183–186	...	Br	25.93	24.78	...
4′,5-Br_2	2 Recr. D	223–225 decompn.	...	Br	41.29	41.09	...
3,4′,5-Br_3	2 Recr. P	196–206	...	Br	51.45	50.95	...
N-Benzylsalicylamides							
3,5-Br_2-4′-Cl	1b Recr. C	139–142	...	[g]	1.237	1.224	...
3,5-Br_2-3′,4′-Cl_2	1b Recr. C	157–160	...	[g]	1.459	1.443	...
Schiff's Bases							
Amine / Aldehyde							
4-Amino-salicylic acid / 5-Bromo-salicylic-aldehyde	4	193–195	194 (35)	Br	23.77	24.64	(35)
4-Bromo-aniline / Salicyl-aldehyde	4 Recr. B	110–112	...	Br	28.94	29.26	...
4-Bromo-aniline / 5-Bromo-salicyl-aldehyde	4 Recr. A	173–176	...	Br	45.02	45.44	...
2-Hydroxy-aniline / 5-Bromo-salicyl-aldehyde	4	186–189	...	Br	27.26	27.11	...
Other Compounds							
2-Amino-3,4′,5-tribromobenz-anilide	5	215–218	...	Br	53.40	52.85	...
4,4′-Dibromo-3-hydroxy-2-naphthanilide	1b Recr. C	230–237 decompn.	234 (32)	Br	37.95	36.69	(32)
3,5-Dibromosalicylamide	2 Recr. B	182.5–184	182–183 (33)	Br	46.87[f]	46.62	(33)
4′-Bromo-2-mercaptobenz-anilide[n]	1b[m]	238–241	...	Br	26.01	24.91	...
N,N′-Hexamethylene bis (3,5-dibromosalicylanilide)	1b Recr. P	204–224	...	Br	47.56	47.50	...

[a] Methods of preparation are numbered as in the specific examples given in the text. [b] Methods of purification include: evaporation of solvent from a solution of the product in a beaker followed by mechanical removal of the top, colored layer from the walls of the beaker (designated "Pur"), and recrystallization (Recr.) from the following solvents: P, chlorobenzene; A, acetone; B, ethanol; C, acetic acid; D, acetone and chloroform; E, ethyl acetate; F, benzene and petroleum ether; T, toluene. [c] Halogenated salicylanilides are listed in the following order: (*a*) bromine derivatives, from mono- to penta-brominated; (*b*) chlorine derivatives, from mono- to tetra-chlorinated; (*c*) bromochloro derivatives (from di- to penta-halogenated, within each group compounds with larger number of bromine substituents are given first; (*d*) a bromofluorosalicylanilide. [d] Microanalyses performed by Schwarzkopf Microanalytical Laboratory, all other analyses were performed by Messrs. W. H. Adam, L. F. Pierro, C. N. Buffa, D. P. Prescott, R. L. Eichenlaub, and D. J. Berry at Lever Brothers Research Center. [e] Second crop, after preliminary filtration of small initial precipitate. [f] Infrared spectrum identical to commercial 3′,4′,5-trichlorosalicylanilide manufactured by Geigy Chemical Co. [g] Grams of silver halide precipitate per gram of sample. [h] Appeared to be dimorphic. [i] In the presence of pyridine. [j] A 25-ml. quantity of chlorobenzene used per Gm. of solid. Crude product obtained by evaporating solvent. [k] In the presence of iodine. [l] One molecule of ethanol of crystallization. [m] In anisole. [n] As the disulfide.

pounds. In some cases when the site available for bromination seemed unambiguous from previous experience, elemental analysis alone served as a basis for identification of the brominated product.

BIOLOGICAL ACTIVITY

Data from the laboratories of Geigy Chemical Co. (1–4), Knoll, A. G. (5–8), Ciba (9–10), BASF (11), Monsanto Chemical Co. (12–14), Wallace and Tiernan (15), and Ben Venue Laboratories (16), as well as several university laboratories (17–19), have illustrated the germicidal activity of a large number of halogenated salicylanilides. This work may be summarized briefly as follows: (*a*) Good activity (bacteriostasis of *S. aureus* 209 at 2 parts of germicide in 1,000,000) could be achieved with substitution in just one ring of salicylanilide. Maximum activity, however, was always associated with substi-

TABLE II.—BROMINATION OF SALICYLANILIDES

Substituents in Starting Material	Moles, Br_2	Solvent	Added Substituents	Method of Identification
None	1	HOAc	5-Br	IR,[a] M.P.
None	2	HOAc	5-Br 4′,5-Br_2[b] 3,4′,5-Br_2	IR
2′-Cl	1	HOAc	5-Br	Anal.
2′-Cl	2	HOAc	3,5-Br_2	IR, M.P.
4′-Cl	1	HOAc	5-Br	M.P.
2′,5′-Cl_2	3[c]	HOAc	3,5-Br_2	IR
3′,5-Br_2	1	HOAc/I_2	3-Br 50% 4′-Br 50%	M.P. M.P.
2′-Br-5-Cl	1	HOAc	Mixtures	M.P.
2′,4′,5-Br_3	1	aq. HOAc	3-Br	IR
2′,3,5-Br_3	1	aq. HOAc	4′-Br	IR
3,3′,5-Br_3	1	HOAc/I_2 long heating	4′-Br	Anal.
3,5-Br_2-3′-Cl	1	aq. HOAc	4′-Br	Anal.
3,5-Br_2-2′,5′-Cl_2	1	aq. HOAc	None	IR
3,5-Br_2-2′-SO_2NH_2	1	aq. HOAc	None	Anal.
3-Me-4′-Br	1	HOAc	5-Br	Anal.
3-Me-3′,5-Br_2	1	HOAc/I_2	4′-Br	Anal.
3′-Me-3,5-Br_2	1	HOAc	4′-Br	Anal.
3,4′-Me_2	1	HOAc	5-Br	Anal.

[a] We are indebted to Dr. S. Goldwasser and Mr. A. Rapisarda of this laboratory for interpretation of infrared spectrogrrms. [b] Major product. [c] Only two moles reacted.

tution in each ring. Dilution values of 1 p.p.m. and better were frequently attained. (*b*) Substitution in the electron-rich positions (*ortho* or *para* to the phenol and amide groups) yielded active compounds as did vicinal substitution (simultaneous *meta* and *para* substitution in either ring). (*c*) Great and apparently unpredictable changes in activity resulted from changing a bromine atom to a chlorine atom in a given structure.

In the present work the biological activity of the test compounds was determined in the filter paper disk (FPD) and skin disk substantivity (SDS) tests previously described (20). Ratings given in these tests have the following significance:

Filter Paper Disk Test.—++, zone greater than 1 mm.; +, 1 mm. zone or less; ±, growth inhibition under disk; 0, no activity.

SDS Test.—Rating of 4, sharply outlined clear area free of growth (excellent activity); rating of 3, clear area of no growth with hazy periphery (good activity); rating of 2, area showing partial growth (fair activity); rating of 1, area showing growth almost equal to surrounding agar (slight activity); rating of 0, area showing growth equal to surrounding agar (no activity).

In the SDS test, six disks are judged and the results averaged numerically to two significant figures which are further rounded off as follows:

SDS TEST SCORES

Average of Six Observations	Score Tabulated
3.6–3.8	4−
3.2–3.5	3+
2.9–3.1	3

Both tests are carried out in the presence of soap or detergent. The SDS test scores depend not only on germicidal activity but also, as the name implies, on the substantivity of the test compound to skin. They might therefore be expected to differ from results previously published in the literature. Nonetheless, the three generalizations listed at the beginning of this section were, in the main, supported by the present results. Thus, substitution in both salicylanilide rings was necessary (but not sufficient) for a score of 3 or better in the SDS test in the presence of soap. The twelve compounds which scored the maximum of 4 were characterized by *ortho* and *para* and/or vicinal substitution. Three of the twelve had only *ortho* and *para* substitution; three possessed vicinal substitution in one ring and were mono-substituted in the other; and six compounds carried *ortho* and *para* substituents in one ring and vicinal substituents in the other.

Interchange of bromine and chlorine had some effect on SDS test scores but the effect is less dramatic than occasionally found in simple dilution tests. Thus, 2′,4′,5-trichlorosalicylanilide scores 2+ and a bromine analog, 5-chloro-2′,4′-dibromosalicylanilide, attains a score of 4. In the usual dilution tests these two compounds have been reported to differ by a factor of 500 in threshold value (5). On the other hand, three out of four 4′,5-dihalosalicylanilides gave identical SDS test scores of 3+; the fourth, 5-bromo-4′-chlorosalicylanilide, scored 3−. Surprisingly, this last compound was the most active one in the dilution test (5). For the eight 3,4′,5-trihalosalicylanilides the scores ranged from 2+ to 4, with six falling between 3 and 4−.

Of the ninety-two test compounds, SDS test scores of 3 or better were recorded for 34 compounds in the presence of soap, for 19 compounds in the presence of an alkaline soap/detergent bar, and for six compounds in the presence of a neutral detergent bar. While fewer compounds were tested in the presence of the latter two bar stocks, it is safe to conclude that high germicidal activity in the presence of detergents is considerably rarer than high activity in the presence of soap.

In general, high activity at 0.5% was associated with structures halogenated in the following positions: 4′,5; 2′,4′,5; 3,4′,5; 3′,4′,5; 4,4′,5; 2′,3,4′,5; 3,3′,4′,5; 2′,3,4′,5,5′.

Substitution by groups other than halogen had a generally depressing effect upon germicidal activity.

TABLE III.—ACTIVITY OF BACTERICIDES AT 0.5% IN SOAP OR NONSOAP DETERGENTS AGAINST *S. aureus*

Substituent and Position	FPD Test[a] in Presence of Bar Stock Type A[b]	SDS Test[a] in Presence of Bar Stock Type A[b]	Type B[c]	Type C[d]
Halogenated Salicylanilides				
4′-Br	−	0+	...	...
5-Br	−	0+	...	...
2′,5-Br_2	...	1	...	...
3,4′-Br_2	0	1+	...	...
3,5-Br_2	±	0+	1	...
3′,5-Br_2	++	3−	1	...
4′,5-Br_2	++	3+	2	1
2′,3,5-Br_3	++	1+	...	<1
2′,4′,5-Br_3	++	3+	2	...
3,3′,5-Br_3	++	2+	2	1
3,4′,5-Br_3	++	3+	3+	1+
3′,4′,5-Br_3	++	4	3	...
4,4′,5-Br_3	++	4	3+	1
2′,3,4′,5-Br_4	++	3+	3+	1+
3,3′,4′,5-Br_4	++	4	3+	3
2′,3,4′,5,5′-Br_5	++	4	...	2
4′-Cl	−	0+	...	...
5-Cl	+	1+	...	...
2′,5-Cl_2	...	1−	...	...
3′,4′-Cl_2	...	0	0	0
3,5-Cl_2	...	0+	...	...
4′,5-Cl_2	++	3+	1+	1
2′,4′,5-Cl_3	++	2+	...	2+
3,3′,5-Cl_3	++	3	...	1−
3,4′,5-Cl_3	++	3+	3	1
3′,4′,5-Cl_3	++	3+	3+	1
2′,3,4′,5-Cl_4	++	4	3+	2
3,3′,4′,5-Cl_4	++	4	4	4
2′-Br-5-Cl	++	0	...	...
4′-Br-5-Cl	++	3+	2	1
5-Br-2′-Cl	...	2−	...	...
5-Br-4′-Cl	++	3−	2	...
2′,4′-Br_2-5-Cl	...	4	...	3−
3,4′-Br_2-5-Cl	++	4−	3	...
3,5-Br_2-2′-Cl	++	1+	1	...
3,5-Br_2-3′-Cl	++	1+	1+	...
3,5-Br_2-4′-Cl	++	2+	3	...
4′,5-Br_2-2′-Cl	++	3+	...	...
4′,5-Br_2-3-Cl	++	4	3	...
3-Br-4′,5-Cl_2	...	3+	4−	...
4′-Br-3,5-Cl_2	++	3	4−	...
5-Br-3,4′-Cl_2	...	3+	4−	...
5-Br-3′,4′-Cl_2	++	4	...	...
3,4′,5-Br_3-3′-Cl	++	4	...	3+
3,5-Br_2-2′,4′-Cl_2	++	4−	3+	1+
3,5-Br_2-2′,5′-Cl_2	++	3	1	...
3,5-Br_2-3′,4′-Cl_2	++	4	...	4
4′-Br-3,3′,5-Cl_3	...	4−	...	4−
3,4′,5-Br_3-2′,5′-Cl_2	++	4	4	4
3,5-Br_2-4′-F	...	1−	...	...
Halogenated Sulfur Derivatives of Salicylanilide				
3,5-Br_2-2′-SO_2NH_2	−	0	...	...
3,4′,5-Br_3-2′-SO_2NH_2	++	1	...	...
3,5-Br_2-4′-SO_2NH_2	−	0	...	...
3,5-Br_2-2′-SH[e]	...	0	...	...
Halogenated Homologs of Salicylanilide				
3,4′,5-Br_3-N-CH_3	−	0	0	
4′-Br-3-CH_3	−	0	...	...
3′,5-Br_2-3-CH_3	++	1	...	...
4′,5-Br_2-3-CH_3	++	1	...	...
3′,4′,5-Br_3-3-CH_3	+	1	...	...
5-Br-2′,4′-Cl_2-3-CH_3	++	3	...	1
5-Br-2′,5′-Cl_2-3-CH_3	++	2	...	1−
3,5-Br_2-3′-CH_3	++	1	...	...
3,4′,5-Br_3-3′-CH_3	++	4−	4	1+
3,5-Br_2-4′-CH_3	++	0	...	...
5-Br-3′-Cl-4′-CH_3	...	2−	...	0+
3,5-Br_2-3′-Cl-4′-CH_3	...	3+	3+	1−
5-Br-3,4′-$(CH_3)_2$	−	0	...	...

TABLE III. (*continued*)

Substituent and Position		FPD Test[a] in Presence of Bar Stock Type A[b]	SDS Test[a] in Presence of Bar Stock Type A[b]	Type B[c]	Type C[d]
Other Halogenated Salicylanilides					
5,5′-Cl_2-2′-OCH_3		++	...	0	...
4′-Br-4-$NHCOCH_3$		−	0	0	...
5-Br-3′,4′-Cl_2-3-C_6H_5		...	2+	...	0
4′,5-Br_2-O-Acetyl		−	...	...	...
3′,4′-Cl_2-3-NO_2		++	1−	...	...
3′,4′-Cl_2-5-NO_2		+	1+	...	1−
5-Br-3′,4′-Cl_2-3-NO_2		++	3+	...	...
4-Hydroxybenzanilides					
4′-Br		−	...	...	...
3,4′-Br_2		−	0	...	...
3,5-Br_2		−	...	...	...
3,4′,5-Br_3		−	0	...	...
Resorcylanilides					
4′-Br		−	0	...	...
4′,5-Br_2		−	0	...	...
3,4′,5-Br_3		−	0	...	...
N-Benzylsalicylamides					
3,5-Br_2-4′-Cl		++	1	...	...
3,5-Br_2-3′,4′-Cl_2		++ (1%)	1 (1%)	...	...
Schiff's Bases					
Amine	Aldehyde				
4-Amino-salicylic acid	5-Bromosal-icylalde-hyde	−	0	...	...
4-Bromo-aniline	Salicylal-dehyde	−	0	...	...
4-Bromo-aniline	5-Bromosal-icylaldehyde	−	0	...	...
2-Hydroxy-aniline	5-Bromosal-icylaldehyde	−	0	...	...
Other Compounds					
2-Amino-3,4′,5-tri-bromobenzanilide		−	...	...	...
4,4′-Dibromo-3-hydroxy-2-naphthanilide		−	1+	...	...
4′-Bromo-2-mercapto-benzanilide[e]		−	0 (1%)	...	...
3,5-Dibromosalicyl-amide		−	...	...	...
N,N-Hexamethylenebis-(3,5-dibromosalicyl-amide)		++	0	...	...

[a] See Ref. 20. [b] Soap bar stock, pH 10.2. [c] A soap/detergent bar stock, pH 9.2. [d] A detergent bar stock, pH 7.0. [e] As the disulfide.

Thus, none of the various thiol or sulfonamide derivatives showed more than moderate activity. The presence of a hydroxyl group in the 4-position destroyed activity whether or not the hydroxyl in the 2-position was retained. Esterification of the phenolic hydroxyl destroyed activity altogether as did the methylation of the nitrogen.

Simple alkylation of one of the benzene rings did not depress the test scores. Thus, 3′-methyl-3,4′,5-tribromosalicylanilide was as active as 3,4′,5-tribromosalicylanilide.

Of the substituents investigated only the nitro group appeared to approach the halogens in the ability to confer germicidal activity upon the salicylanilide structure. Thus, 3′,4′ - dichloro - 5-nitrosalicylanilide is marginally more active than the corresponding 5-chloro or 5-bromo compounds.

Structures analogous to salicylanilide such as Schiff's bases derived from salicylaldehyde, resorcylanilides, 4-hydroxybenzanilides and N-benzylsalicylamides showed little or no germicidal activity.

Table III summarizes the biological activity of the test compounds.

CONCLUSIONS

Germicidal activity and skin substantivity of halogenated salicylanilides are influenced by the test medium. Of the ninety-two compounds here

described, fewer were found to be active in the presence of a neutral detergent than in the presence of an alkaline soap.

In a soap base, high germicidal activity and skin substantivity of salicylanilides is associated with halogenation in the following positions: 4′,5; 2′,4′,5; 3,4′,5; 3′,4′,5; 4,4′,5; 2′,3,4′,5; 3,3′,4′,5; 2′,3,4′,5,5′.

In soap/detergent bar stock of intermediate pH several of the products are less active. In particular, all of the 4′,5-dihalosalicylanilides have SDS test scores of less than 3 in this medium. In neutral detergent bar stock only the 3,3′,4′,5 - tetrahalosalicylanilides and 2′,5′-dichloro - 3,4′,5 - tribromosalicylanilide exhibit appreciable activity.

Substitution of salicylanilide with groups other than halogens or modification of the salicylanilide structure usually diminishes the activity of these germicides. Only certain homologs and nitro-derivatives of the halosalicylanilides show high activity. Ring substitution with hydroxyl, methoxyl, thiol, or sulfonamide groups affects the activity adversely. Germicidal activity is destroyed completely by methylation of the amide nitrogen or by acetylation of the phenolic hydroxyl. Halogenated salicylanilides possess higher germicidal activity and skin substantivity than analogous derivatives of N-benzylsalicylamide, salicylnaphthanilides, or Schiff's bases of salicylaldehyde.

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Spectrophotometric Assay of Opium and Paregoric

By CHARLES MILOS

A method is proposed for the determination of the morphine content of opium and paregoric. The method combines extractive and spectrophotometric procedures and results in a substantial saving of time when compared to most existing methods. Results thus far obtained indicate that the method is sufficiently accurate for routine analyses.

THE DETERMINATION of morphine in opium by the U. S. P. method requires a preliminary extraction with water, evaporation of the filtrate, addition of calcium hydroxide and filtration, precipitation, and titration of the morphine (1). Most other common methods require an initial extraction of the opium with lime water and subsequent precipitation or extraction of the morphine from an aliquot of the lime water filtrate. All of these methods are long, involved, and require considerable experience to secure consistent results. There is no general agreement as to what method gives the most accurate value for the morphine content of opium. As a matter of fact, all of the methods probably have sources of error; and determinations made by different methods usually show considerable variation. Buchi, Huber, and Schumacher have

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investigated and discussed the probable sources of error for various methods of determining morphine in opium (2).

A shorter and more convenient method for the determination of morphine in opium would be of obvious value in the routine analyses of opium to determine quality or conformity to standards. Such a method should, of course, give reproducible results in approximate agreement with accepted methods. The present study, therefore, was directed toward the development of a shorter, more convenient method, rather than the development of a method claiming more exact representation of morphine content. It was considered desirable that the method should be applicable also to the determination of morphine in paregoric, the most common liquid preparation of opium.

Some procedure involving spectrophotometric determination of morphine seemed a possible solution of the problem; for the absorption spectra of morphine in acid and alkaline solutions have been reported by numerous observers (3–7). Obviously it would be necessary to secure a solution of the morphine reasonably free from color, resins, and other alkaloids present in the opium. Some extractive procedures were therefore unavoidable; and so the primary effort was directed toward the development of a method combining extractive and spectrophotometric procedures, which would materially shorten existing methods. The following method was eventually developed.

METHOD

Reagents.—Acetic acid solution, 2% aqueous solution; barium acetate C. P.; isobutyl alcohol C. P.; chloroform U. S. P.; ammonium hydroxide solution, dilute 1.0 ml. of ammonium hydroxide (sp. gr. 0.9) to 100 ml. with distilled water; alkaline salt solution, dissolve 3 Gm. of sodium hydroxide in 100 ml. of a saturated solution of sodium chloride and filter; sulfuric acid solution, approx. 2 *N*; sulfuric acid solution, 0.1 *N*; potassium hydroxide solution, aqueous solution containing 50% by weight of potassium hydroxide.

Procedure.—Accurately weigh 1 Gm. of opium and transfer to a 250-ml. beaker. Add 50 ml. of 2% acetic acid solution and heat just to boiling for about four minutes. During the heating, macerate the opium into fine particles using a glass rod. Allow to settle and filter into a 250-ml. volumetric flask, pouring the liquid from the beaker carefully to retain as much of the opium as possible in the beaker. A Whatman No. 12 folded filter paper is convenient for the filtration. Repeat the operation, using another 50-ml. portion of 2% acetic acid solution, heating and filtering as before. When the second filtration is complete, transfer the filter paper to the beaker, add 50 ml. of the acetic acid solution, heat as before, and filter through a fresh filter paper into the 250-ml. flask. Again add 50 ml. of the acetic acid solution to the beaker, heat, and filter. The final portion of filtrate should be practically colorless. Cool the combined filtrations in the 250-ml. volumetric flask and make up to volume with distilled water.

Pipet 10 ml. of the filtrate from the 250-ml. flask into a small porcelain dish, add approximately 300 mg. of barium acetate, and evaporate to dryness on a steam bath. To the residue in the dish add 10 ml. of isobutyl alcohol, stir with a glass rod while the solvent becomes hot, and filter the hot isobutyl alcohol into a separator. For this filtration, use a Whatman No. 12, 12.5-cm. folded filter paper. Leach the residue in the dish twice more with 5-ml. portions of hot isobutyl alcohol. Filter these solvent portions through the same filter paper into the separator.

To the combined isobutyl alcohol in the separator add 50 ml. of chloroform and shake to obtain a uniform solution. Add 10 ml. of the dilute ammonium hydroxide solution to the separator and shake for a few minutes. After complete separation of the two layers, draw off the chloroform-isobutyl alcohol into another separator. Extract the aqueous solution remaining in the first separator with a 5-ml. portion of chloroform and add this chloroform to the chloroform-isobutyl alcohol in the second separator. Discard the aqueous solution of the first separator. To the chloroform-isobutyl alcohol solvent in the second separator add 15 ml. of alkaline salt solution and shake for a few minutes. Allow the two layers to separate thoroughly, then draw off and discard the lower solvent layer. Extract the alkaline salt solution remaining in the separator with a 10-ml. portion of chloroform; draw off and discard the chloroform. Neutralize the alkaline salt solution in the separator with approximately 2 *N* sulfuric acid, using litmus paper as indicator; then add approximately 1 ml. excess of the 2 *N* acid solution in order to make the solution roughly 0.1 *N*. Transfer the acid solution to a small beaker. Wash the separator with a small portion of 0.1 *N* sulfuric acid solution and add this to the beaker. Place a few glass beads in the beaker and heat the solution on a steam bath to remove any remaining chloroform. Stirring occasionally with a glass rod will facilitate the escape of the chloroform. Cool the solution in the beaker, transfer to a 100-ml. volumetric flask (filter if not perfectly clear), and make up to volume with 0.1 *N* sulfuric acid solution, using a few portions to rinse the beaker.

By means of a pipet, transfer 3 ml. of the final acid solution into a 1-cm., clean, dry silica cell. To a matched silica cell add 3 ml. of 0.1 *N* sulfuric acid solution for use as a reference blank. Determine the absorbance of the sample solution at 300 mμ, with the spectrophotometer at full sensitivity, zero suppression, and a slit opening of not more than 0.08 mm. Now add exactly 0.5 ml. of 50% potassium hydroxide solution to the sample cell and to the reference cell. Mix, and again determine the sample absorbance at 300 mμ, using the solution in the reference cell as a blank.

$$[(A300b \times 1.167) - A300a] \times 12.2 = \text{mg. anhydrous morphine per 100 ml. of solution determined}$$

where $A300b$ = absorbance of alkaline solution at

300 mμ, $A300a$ = absorbance of acid solution at 300 mμ, 1.167 = correction for dilution with potassium hydroxide solution, 12.2 = conversion factor for readings.

$$\frac{\text{mg. anhyd. morphine (from above)} \times 25}{\text{wt. sample, mg.}} \times 100 = \%\text{ by wt. of morphine in opium}$$

Paregoric.—For the assay of morphine in paregoric by this method, pipet 10 ml. of the paregoric into a small porcelain dish, add about 300 mg. of barium acetate, and proceed as in the assay for opium from that point.

DISCUSSION

The proposed method employs a preliminary extraction of the opium similar to that of the U. S. P. except that dilute acetic acid solution rather than plain water is used. The addition of barium acetate to the filtrate renders the resins and coloring matter less soluble in the isobutyl alcohol used to extract the morphine from the residue. Other alkaloids in the opium are, of course, extracted by the isobutyl alcohol; but these are separated from the morphine by the subsequent extraction with alkaline salt solution.

Morphine shows a maximum absorption in alkaline solution at 298 mμ. From 298–301 mμ, inclusive, the absorption of morphine in alkaline solution obeys Beer's law. The extinction coefficient, $E(1\%, 1\text{ cm.})$, for morphine in alkaline solution at 300 mμ was found to be 81.96. In 0.1 *N* sulfuric acid solution, morphine shows no absorption at 300 mμ. The proposed method takes advantage of the fact that morphine in acid solution shows no absorption at 300 mμ but does show absorption in alkaline solution because of the bathochromic shift of the morphine molecule.

Opium contains a number of compounds which show absorption at 300 mμ in both alkaline and acid solution. Some of these compounds are alkaloids; others are nonalkaloidal (8). The proposed method, for practical purposes, removes all of the alkaloids except morphine. In respect to the nonalkaloidal compounds, experiments by the author indicate that a very small amount of a substance, or substances, showing absorption at 300 mμ, does accompany the morphine in the proposed method. However, the absorption of this substance shifts so slightly in alkaline solution that for practical purposes its absorption in alkaline and acid solution may be considered equivalent. Hence, it should not materially affect the accuracy of the proposed method.

The determination of morphine in opium by the proposed method requires a maximum of four hours for completion. For paregoric the time required is appreciably less, since the initial extractions with dilute acetic acid are unnecessary.

ANALYTICAL RESULTS

Table I shows results obtained for various samples of opium by the proposed spectrophotometric method and by the U. S. P. method. Results secured by an extraction method are also shown. For the extraction procedure, 50 ml. from the initial filtrate (dilute acetic acid solution) of the present method was assayed according to the U. S. P. method for paregoric.

TABLE I. RESULTS OBTAINED FOR VARIOUS SAMPLES OF OPIUM[a]

Type of Opium	U. S. P. Method	Extraction Method	Proposed Spectrophotometric Method
Pakistan	7.5	8.9	7.8, 7.6, 7.4
Indian	10.5	12.0	11.4, 11.2
Turkish	12.8	...	13.8
Jugoslav	...	14.5	13.6
Turkish	...	13.1	12.5

[a] Morphine, % by weight.

TABLE II.—RESULTS OBTAINED ON SAMPLES OF PAREGORIC[a]

Sample	U. S. P. Assay	Spectrophotometric Assay
1	37.8	38.8, 37.9, 39.2, 39.6
2	...	38.7
3	...	36.0
4	...	37.4
5	...	40.8

[a] Morphine, mg. per 100 ml.

Table II shows results obtained on samples of paregoric by the proposed method. Sample No. 1 in the table represents a sample of paregoric prepared by the author according to U. S. P. specifications. The morphine content of this sample was determined by the official U. S. P. procedure and by the proposed spectrophotometric method (four determinations). Samples 2, 3, 4, and 5 in the Table represent commercial samples of paregoric presumably made according to U. S. P. specifications (35–45 mg. of morphine per 100 ml.).

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Biological Disposition of Apomorphine IV

Isolation and Characterization of "Bound" Apomorphine

By P. N. KAUL†, E. BROCHMANN-HANSSEN, and E. LEONG WAY

"Bound" apomorphine, the major metabolic product of apomorphine, has been isolated from the urine of rabbits by a series of ion exchange procedures, fractionated by paper chromatography, and identified by enzymatic hydrolysis and various color tests. It has been found to consist of two metabolites which, based on electronic absorption and stereochemical consideration, are designated as apomorphine 3,O- and 4,O-β-glucuronides, respectively.

PREVIOUS STUDIES indicated that apomorphine is excreted largely in its "bound" form (1). The rate of formation of the "bound" apomorphine and the site of its biotransformation have already been reported (2). This paper reports the isolation and characterization of "bound" apomorphine which constitutes the major metabolic product of the drug.

EXPERIMENTAL

Purification and Isolation of "Bound" Apomorphine.—Several rabbits were given 10 mg./Kg. of apomorphine hydrochloride daily for several days, and the urine samples collected were pooled. The main bulk was concentrated with the help of a rotary vacuum evaporator to about half the original volume. Portions of this concentrated solution containing the crude "bound" apomorphine were subjected to various procedures for purification and isolation.

Based on analogies from other phenolic compounds which are known to be excreted as ethereal sulfates or glucuronides (3), it would seem reasonable to assume that apomorphine is conjugated at either or both phenolic hydroxyl groups. It should, therefore, have elements of a dissociable nitrogen and an acidic group, functions which lend themselves to the application of ion exchange techniques. A 10-ml. sample of concentrated urine (apomorphine equivalent 0.17%) was passed through a 1×8 cm. column of Dowex 50-X2 (H^+) (50–100 mesh). The column was washed with water, alcohol, and again with water. This treatment removed most of the colored and turbid impurities. The "bound" apmorphine retained on the column was eluted with 4 *N* ammoniacal methanol. The eluate was evaporated to dryness under reduced pressure, and the residue was dissolved in water. The residue from an aliquot of this solution had an apomorphine equivalence of 3.3%, indicating about twentyfold concentration over the starting material.

The partly purified solution was basified with ammonia and passed through a Dowex 1-X8 (50–100 mesh) formate column (1 × 8 cm.). Such columns have been effectively used for the separation of sugar phosphates (4) and for uronides of biochemical significance (5). The column was washed with water and the "bound" apomorphine eluted with 0.2 *N* formic acid. The eluate, which gave positive tests for apomorphine after acid hydrolysis, was evaporated to dryness under reduced pressure. The residue was dissolved in 50% ethanol and again evaporated to dryness under reduced pressure. This procedure was repeated in order to remove traces of formic acid. Finally, the residue was dissolved in 50% alcohol and analyzed (1). It showed an apomorphine equivalence of 20%, indicating an overall concentration of about one hundred and twentyfold over the starting material. Further purification of the isolated compound was possible by evaporating the solution to dryness and taking up the residue in water. Some colorless solid remained undissolved. On repeating several times the evaporation and the redissolving of the residue in water, the purity of the isolated "bound" apomorphine was increased three hundredfold. The final solution yielded a residue with an apomorphine equivalence of 46%.

Nature of the "Bound" Apomorphine.—A concentrated solution of the "bound" apomorphine, isolated by the ion exchange procedures, was analyzed by descending chromatography on Whatman No. 1 paper and with a solvent system composed of butanol, acetic acid, water (5:1:4). When the chromatograms were exposed to ultraviolet light, two fluorescent spots appeared. These spots acquired an orange color when sprayed with Dragendorff's reagent, indicating the presence of substances of an alkaloidal nature. From a series of chromatograms, the two fluorescent spots were cut out and eluted separately with water. The eluates from corresponding spots were combined and concentrated under reduced pressure to a small volume. Neither of the two resulting solutions gave tests for free apomorphine. However, both yielded apomorphine on acid hydrolysis, suggesting that apomorphine was being excreted in urine as two conjugated metabolites. The upper spot (lower R_f value) accounted for about 70% of the total concentration of "bound" apomorphine.

The solutions of the two conjugates were hydrolyzed separately with 2 *N* hydrochloric acid at reflux

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temperature for forty minutes. This acid strength was used because, at the acid concentration of 4 *N* used in the procedure described for estimation of "bound" apomorphine (1), glucuronic acid is decomposed. The hydrolyzates were passed through a column of Amberlite IR 4-B, a weakly anionic exchanger, to remove the mineral acid, and then through a column of Dowex 50 (H^+) in order to remove apomorphine. The colorless solutions thus obtained were evaporated under reduced pressure to small volumes. Both gave positive tests for reducing sugars with Fehling's solution.

The solutions of the reducing substances isolated above were chromatographed on Whatman No. 1 filter paper alongside authentic glucuronic acid and glucuronolactone. Butanol, acetic acid, water (5:1:4) was used as the descending solvent system. The chromatograms were dried and sprayed with *m*-phenylenediamine dihydrochloride, aniline phthalate, and naphthoresorcinol reagents.

Two spots were observed from both the isolated reducing solutions with all the three reagents. The reducing spot of higher R_f value (0.37) compared with that of authentic glucuronolactone and the spot of lower R_f value (0.18) compared similarly with authentic glucuronic acid. The appearance of two spots indicated that at the pH at which the solution was spotted, the sugar acid existed as a mixture of free acid and lactone. It thus appeared that both components of the "bound" apomorphine were glucuronides. This was further confirmed by enzymatic hydrolysis of the metabolites as follows: 1 ml. each of the solutions, containing about 500 mcg. of the "bound" metabolites, were treated in separate flasks with 0.5 ml. of 0.4 *N* acetate buffer of pH 5 and 1 ml. of β-glucuronidase solution (250 Fishman units). In another pair of flasks, the two metabolite solutions were mixed with the buffer solution while the enzyme was omitted. Finally, in a fifth flask 1 ml. of an aqueous solution of authentic phenolphthalein glucuronide was treated with 0.5 ml. of acetate buffer and 1 ml. of the enzyme solution. The last flask served as a control in order to check the activity of the enzyme preparation. At every sixth hour, an additional 0.5 ml. of the enzyme solution was added to the flasks which orginally contained the enzyme. All the flasks were incubated at 37 ±1° for twenty-four hours, after which the solutions were analyzed for the products of hydrolysis.

The solutions of the flasks containing the "bound" metabolites and the enzyme gave positive tests for free apomorphine, whereas the flasks containing the "bound" metabolite without the enzyme did not show any hydrolysis of the "bound" metabolites under the experimental conditions. The control flask contained free phenolphthalein, indicating the effectiveness of the enzyme preparation.

Structural Characterization of Two Apomorphine β-Glucuronides.—Since apomorphine has two phenolic groups, there is a possibility of either one or both being conjugated with glucuronic acid. To ascertain whether the metabolites are mono- or di-conjugates of glucuronic acid, the aqueous solutions of the isolated apomorphine glucuronides were subjected to ultraviolet absorption analysis.

Figure 1 (solid line) shows the spectrum of the glucuronide occurring in higher proportion. It is similar to that of apomorphine, except for the fact

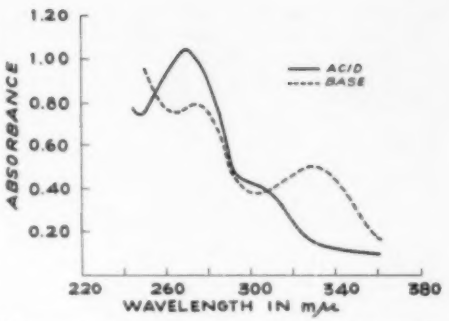
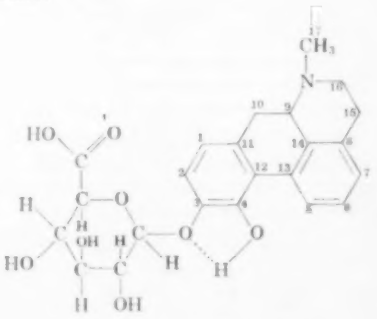


Fig. 1.—pH-dependent reversal of ultraviolet absorption spectrum of "bound" apomorphine.

that the peak occurs at 270 instead of at 272 mμ. When a drop of 6 *N* sodium hydroxide solution was added to the solution of this glucuronide, a bathochromic shift with a peak at 330 mμ was observed (Fig. 1, dotted line). On addition of two drops of 6 *N* hydrochloric acid to the same cell, the spectrum reverted back to the original solid curve.

The ultraviolet spectrum of the metabolite occurring in lower proportion (30%) was very similar to the one just described, except that the peak occurred at 267 mμ. The same bathochromic shift was observed upon addition of alkali, with a return to the original spectrum after acidification.

This reversible pH-dependence of the electronic absorption indicated that there was a free phenolic group present in both metabolites. They must, therefore, be mono-glucuronides. Steric consideration of the apomorphine molecule reveals that the 3-position is much less hindered than the 4-position, and that the hydroxyl group at 4-position is mostly hydrogen-bonded to the oxygen at 3-position. Hemi-acetal formation with glucuronic acid at 4-position would, therefore, need a higher energy of activation to overcome the energy barrier due to the hydrogen bonding and spatial interaction. Thus, kinetically as well as thermodynamically, glucuronide formation will be favored in the 3- rather than 4-position. This suggests that the major component of "bound" apomorphine is apomorphine 3,O-glucuronide with the following structure:



Apomorphine 3,O-glucuronide

The second component, which constitutes the remaining 30% of the "bound" apomorphine, may be

regarded as apomorphine 4,O-glucuronide. In this structure, the bulk of glucuronic acid becomes proximal to the apomorphine molecule. Due to spatial interaction, this may distort and inhibit the relative coplanarity of the alkaloidal molecule, with the result that resonance of the biphenyl part of the apomorphine molecule would be decreased. This steric inhibition of resonance would explain the hypsochromic shift in the electronic absorption spectrum observed for this molecule.

Since both metabolites were hydrolyzed by β-glucuronidase, an enzyme known to be specific for β-glucosidic linkage, they may be regarded as 3,O and 4,O-β-glucuronides, respectively.

On the basis of evidence presented for the closely related morphine glucuronide (6, 7), the "bound" metabolites of apomorphine may be assumed to exist in the zwitterion form.

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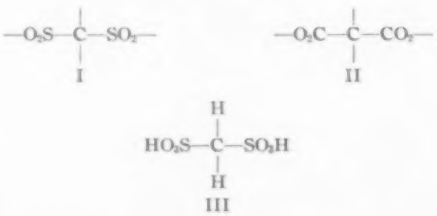
Synthesis of N-Substituted Methanedisulfonamides and N′-Substituted Methanedisulfonylureas

By WAYNE V. KESSLER† and GLENN L. JENKINS

The preparation of various derivatives of methanedisulfonic acid is reported. These include fifteen N-substituted methanedisulfonamides and four N′-substituted methanedisulfonylureas.

SEVERAL INVESTIGATORS (1–6) have observed the similarities between the chemical structure of methanedisulfonic acid and the chemical structures of certain therapeutically useful organic sulfur and malonic acid derivatives. Certain sulfonamides, sulfones, sulfoxides, and sulfides have been used as chemotherapeutic agents (7). Numerous aliphatic sulfone derivatives (I) and malonic acid derivatives (II) have hypnotic properties. The similarities between the chemical structures of these compounds and that of methanedisulfonic acid (III) is apparent. These observations have induced the synthesis of many derivatives of methanedisulfonic acid for pharmacological and chemotherapeutical evaluation (3–6).



I II III

The discovery of the therapeutic value of various sulfur-containing compounds in recent years has intensified the research in this area. The recent preparation of new sulfonylureas (8, 9) for evaluation as hypoglycemic agents and N-substituted alkanesulfonamides from methane- and ethanesulfonic acid (10) for evaluation in the treatment of experimental traumatic shock and as bacteriostatic and bactericidal agents may be cited as examples.

This investigation was conducted with the purpose of synthesizing new derivatives of methanedisulfonic acid which might possess useful pharmacological or chemotherapeutical activity. These derivatives are N-substituted methanedisulfonamides (X) and N′-substituted methanedisulfonylureas (XI).

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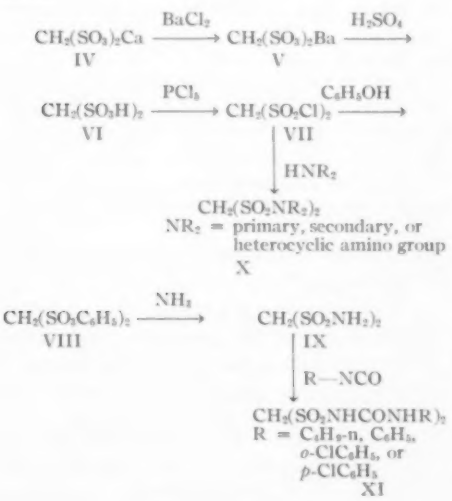
Calcium methanedisulfonate (IV) was available and was used for the starting material. It was converted to the barium salt (V) with barium chloride solution (4), and the barium salt was treated with aqueous sulfuric acid to form methanedisulfonic acid (VI) (2). The product obtained in this manner was sufficiently pure for the preparation of the acid chloride.

The acid chloride (VII) was prepared by the action of phosphorus pentachloride upon the dry acid (2, 11). It was purified by fractional distillation under reduced pressure and was reacted with phenol to form diphenyl methanedisulfonate (VIII) (11). Methanedisulfonamide (IX) was obtained by heating a benzene solution of the diphenyl ester with an excess of liquid ammonia in a bomb (11).

The N-substituted methanedisulfonamides were synthesized from the acid chloride and the appropriate amines (3, 11). Twice the calculated amount of amine was used to serve as the hydrogen chloride acceptor. The N′-substituted methanedisulfonylureas were synthesized by a method similar to that used by Kurzer (12) for the preparation of N-aryl-N′-arylsulfonylureas. The disodium salts of three of these compounds were prepared to facilitate their purification.

The scheme used in this synthesis was as indicated.



EXPERIMENTAL

All melting points reported in this paper are uncorrected and were determined by the open capillary tube method. Nitrogen and sulfur determinations were done by Dr. Alfred Bernhardt.[1] Sodium determinations were done by nonaqueous titrations with acetous perchloric acid.

N-Substituted Methanedisulfonamides.—These compounds were prepared by the following general procedure: In a 500-ml. three-necked, round-bottomed flask, equipped with a mechanical stirrer, dropping funnel, and reflux condenser carrying a calcium chloride tube, were placed 0.4 mole of the appropriate amine and 250 ml. of anhydrous benzene. The stirrer was started and a solution of 0.1 mole of methanedisulfonyl chloride in 75 ml. of anhydrous benzene was added, dropwise, over a period of one hour. After all the acid chloride had been added, the reaction mixture was refluxed and stirred for one to fifteen hours and was then cooled. The sulfonamide was separated from the amine hydrochloride and was then recrystallized from a suitable solvent until a constant melting point was obtained. Data for these compounds are listed in Table I.

Disodium N′-Substituted Methanedisulfonylureas.—These compounds were prepared by procedures similar to that used for the preparation of disodium N,N′-bis-(N-phenylcarbamyl)methanedisulfonamide: In a 100-ml. round-bottomed flask, fitted with a reflux condenser and a mechanical stirrer, were placed 17.4 Gm. (0.1 mole) of methanedisulfonamide, 31.4 Gm. (0.264 mole) of phenyl isocyanate, and 4 ml. of triethylamine. This mixture was heated on a steam bath for twelve hours with stirring. The methanedisulfonamide gradually dissolved, and after about three hours the reaction mixture began to solidify and form a yellow viscous mass. At the end of the reaction, the reaction mixture, while still hot, was dissolved by stirring with 240 ml. of 4% sodium hydroxide solution in divided portions at 70°. The residual insoluble solid was filtered from the hot solution and washed with a small amount of boiling water. These washings were added to the main filtrate. Two grams of decolorizing charcoal was added to the filtrate, and this mixture was stirred at 60° for one-half hour. The mixture was then filtered, and the filtrate was set aside for several hours. The disodium derivative crystallized upon cooling. The filtrate was finally placed in an ice water bath for two hours, after which time the product was collected on a Büchner funnel and recrystallized from an ethanol-water mixture until a constant melting point was obtained. Data for these compounds are listed in Table II.

N,N′-Bis-(N,*n*-butylcarbamyl)methanedisulfonamide.—In a 200-ml. round-bottomed flask, fitted with a reflux condenser and a mechanical stirrer, were placed 26.1 Gm. (0.15 mole) of methanedisulfonamide, 39.7 Gm. (0.4 mole) of *n*-butyl isocyanate, and 6 ml. of triethylamine. This mixture was heated on a steam bath for twelve hours with stirring. The methanedisulfonamide gradually dissolved, and after about one hour the reaction mixture began to solidify and form a yellow mass. At the end of the reaction, the reaction mixture, while still hot, was dissolved in 360 ml. of 4% sodium hydroxide solution. The resulting solution was cooled and extracted with two 100-ml. portions and one 50-ml. portion of benzene. It was then acidified with glacial acetic acid and cooled in an ice water bath for one hour. The product, which precipitated during the acidification, was collected and recrystallized three times from 95% ethanol. The

[1] Mikroanalytisches Laboratorium im Max-Planck Institut für Kohlenforschung, Kaiser Wilhelm Platz 1, Mulheim (Ruhr), Germany.

TABLE I.—N-SUBSTITUTED METHANEDISULFONAMIDES

$R_2N—SO_2CH_2SO_2—NR_2$

NR_2	Formula	Recrystallizing Solvent	Yield, %	M. P., °C.	Nitrogen, % Calcd.	Nitrogen, % Found
Benzylamino[a]	$C_{15}H_{18}N_2O_4S_2$	Dioxane-H_2O	38.1	213.5–214.5	7.90	7.97
1-Phenylethylamino[b,c,d]	$C_{17}H_{22}N_2O_4S_2$	EtOH-dioxane-H_2O	35.6	156 –158	7.32	7.11
2-Phenylethylamino[b,c]	$C_{17}H_{22}N_2O_4S_2$	EtOH-dioxane	26.7	164 –166	7.32	7.21
4-Methylpiperazino[b,c]	$C_{11}H_{24}N_4O_4S_2$	Abs. EtOH	37.9	149 –150	16.46	16.12
2,6-Dimethylmorpholino[b,f]	$C_{12}H_{26}N_2O_6S_2$	Abs. EtOH	54.6	164 –170[g]	7.56	7.62
2,5-Dimethoxyanilino[b,e]	$C_{17}H_{22}N_2O_6S_2$	EtOH-dioxane	75.3[h]	139 –140	6.27	6.20
2,5-Diethoxyanilino[b,h]	$C_{21}H_{30}N_2O_6S_2$	Abs. EtOH	66.9[h]	125 –126	5.57	5.45
2-Methyl-5-nitroanilino[i]	$C_{15}H_{16}N_4O_6S_2$	Dioxane	64.4[h]	243.5–245[m]	12.61	12.51
N-Benzylanilino[b,e]	$C_{27}H_{26}N_2O_4S_2$	EtOH-dioxane	78.7	147.5–148.5	5.53	5.72
N-Ethyl-*o*-toluino[c,d]	$C_{19}H_{26}N_2O_4S_2$	EtOH-dioxane-H_2O	67.7[h]	155 –157	6.82	6.76
N-Ethyl-*m*-toluino[c]	$C_{19}H_{26}N_2O_4S_2$	EtOH-H_2O	61.9[h]	104.5–105.5	6.82	6.53
N-Ethyl-*p*-toluino[c,j]	$C_{19}H_{26}N_2O_4S_2$	EtOH-H_2O	76.1[h]	125.5–127	6.82	7.06
N-Benzyl-*o*-toluino[c,d,j]	$C_{29}H_{30}N_2O_4S_2$	EtOH-dioxane	79.7[h]	206 –207	5.24	5.18
N-Benzyl-*m*-toluino[c,d]	$C_{29}H_{30}N_2O_4S_2$	Abs. EtOH	66.0[h]	144.5–145.5	5.24	5.17
N-Benzyl-*p*-toluino[c,d,j]	$C_{29}H_{30}N_2O_4S_2$	EtOH-dioxane	84.9[h]	170 –171.5	5.24	5.29

[a] The reaction solvent was anhydrous toluene instead of anhydrous benzene. [b] The solution of amine was refluxed while the acid chloride was added. [c] Both the sulfonamide and amine hydrochloride precipitated from the reaction mixture. The sulfonamide was separated as the disodium derivative by recrystallization of the reaction product from 2 *N* sodium hydroxide solution. The free sulfonamide was obtained by acidification of a hot solution of the disodium derivative with concentrated hydrochloric acid. [d] The temperature of the reaction mixture was maintained at about 20° by external cooling during the addition of the acid chloride. [e] The amine hydrochloride precipitate was filtered from the reaction mixture and washed with benzene. The washings were added to the main filtrate and the combined solution was evaporated to dryness to obtain the impure sulfonamide. [f] The impure sulfonamide was washed with several portions of ethanol-water mixture before recrystallization. [g] Both the sulfonamide and amine hydrochloride precipitated from the reaction mixture. The combined precipitate was washed with benzene, air dried, washed with water, and finally dissolved in a minimum amount of acetone. This solution was added to an excess of water and the resulting impure sulfonamide was collected. [h] Neither the amine hydrochloride nor the sulfonamide precipitated from the reaction mixture. The benzene was removed from the reaction mixture and the resulting yellow syrup was washed with ethanol-water mixture to remove amine hydrochloride. [i] Not all the amine hydrochloride precipitated. The benzene was removed from the reaction mixture, the residue was washed with water, the resulting solid was dissolved in a minimum amount of acetone, and this solution was added to an excess of water. The impure sulfonamide was collected and decolorized with charcoal. [j] Both the sulfonamide and amine hydrochloride precipitated from the reaction mixture. The combined precipitate was washed with benzene, air dried, and washed with water to obtain the impure sulfonamide. [k] The solid which precipitated from the reaction mixture was washed with benzene, air dried, washed thoroughly with water acidified with hydrochloric acid, and dissolved in warm 2 *N* sodium hydroxide solution. This solution was extracted with benzene and acidified with concentrated hydrochloric acid. The resulting solid was washed with water and dissolved in a minimum amount of acetone. This solution was added to an excess of water and the resulting impure sulfonamide was collected. [l] A crystal of amine hydrochloride was added to the cool reaction mixture to induce crystallization of the amine hydrochloride. [m] The yield is based on 0.2 mole of amine and 0.05 mole of acid chloride. [n] Softened at 164° and was completely liquid at 170°. [o] With decomposition.

TABLE II.—DISODIUM N′-SUBSTITUTED METHANEDISULFONYLUREAS

$RNHCON(Na)SO_2CH_2SO_2N(Na)CONHR$

R	Formula	Yield, %	M. P., °C.	Sulfur, % Calcd.	Sulfur, % Found	Sodium, % Calcd.	Sodium, % Found
Phenyl	$C_{15}H_{14}N_4O_8S_2Na_2$	23.9	282.5–284[a]	14.05	13.88	10.08	10.21
p-Chlorophenyl	$C_{15}H_{12}Cl_2N_4O_8S_2Na_2$	11.2	284 –284.5[a]	12.21	12.36	8.76	8.78
o-Chlorophenyl	$C_{15}H_{12}Cl_2N_4O_8S_2Na_2$	19.0	245[b]	12.21	12.04	8.76	8.90

[a] With decomposition. [b] Began to sinter above 200° and rapidly turned brown with effervescence above 245°.

yield was 26.1 Gm. (46.8%); m. p. 200.5–202° (with effervescence).

Anal. Calcd. for $C_{11}H_{24}N_4O_6S_2$: N, 15.05; S, 17.22. Found: N, 14.82; S, 16.85.

SUMMARY

1. The objective of this research project was the synthesis of methanedisulfonic acid derivatives for pharmacological and bacteriological evaluation.

2. Fifteen new N-substituted methanedisulfonamides have been synthesized.

3. Four new N′-substituted methanedisulfonylureas have been synthesized.

4. Sufficient quantities of these compounds are available for pharmacological and bacteriological evaluation.

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Sensitive Colorimetric Method for the Determination of Panthenol in Multivitamin Preparation

By ALFRED F. ZAPPALA and CHARLES A. SIMPSON

The described method involves separation of panthenol or pantothenate from its decomposition products and subsequent alkaline hydrolysis. The hydrolysis products are chlorinated and iodine is liberated by addition of an iodide solution. The yellow color formed is stabilized and determined colorimetrically. This method is comparable in time to existing methods and has the advantage of increased sensitivity. The colorimetric part of the procedure is also applicable to other N-H containing compounds.

CONFRONTED WITH the problem of determining panthenol in a multivitamin preparation,[1] the authors initially tried the naphthoquinone method of Schmall and Wollish (1). In this method, ion exchange resins and adsorbents are used for the removal of interfering substances as well as the hydrolysis products of panthenol and pantothenates. Subsequently, the molecule is cleaved with strong base and the resultant β-alanol is coupled with 1,2-naphthoquinone-4-sulfonate and measured colorimetrically.

When the multivitamin preparation used in most of our work was subjected to the method just described, the solution darkened considerably and to varying degrees during the hydrolysis step. The reason for this was determined to be the high concentration of sugars (15 per cent) present in this preparation.

It was thought that a more sensitive method such as the ninhydrin procedure of Schmall and Wollish (*loc. cit.*), would tend to circumvent the problem of high sugar concentration. This method requires the use of cyanide and consequently was felt to be undesirable as it would necessitate some rather involved safety regulations.

The purpose of the present work was to eliminate this interference. One approach was to devise an additional separation. Another approach was to use a more dilute sample solution in conjunction with a more sensitive color reaction, inasmuch as it is known that the chromatographic column is capable of removing moderate amounts of sugars.

EXPERIMENTAL

Separation from Sugars.—Panthenol was quantitatively extracted from the powdered sample with chloroform, and the chloroform-insoluble sugars were removed by filtration. An aliquot of the filtrate was evaporated to dryness, *in vacuo*, and the resulting residue applied to the chromatographic column of Schmall and Wollish (*loc. cit.*) as an aqueous solution. The eluate was then subjected to the naphthoquinone method.

The results obtained using the technique were considerably better than those previously obtained. This procedure, however, is not applicable to liquid multivitamin preparations. Difficulty was encountered in obtaining quantitative extraction with chloroform due to the unfavorable partition between the two phases.

Color Reaction.—The method of Ellis and Hetzel (2) appeared promising, since it is a general method applicable to compounds containing an N—H group. In this procedure, water-soluble N—H compounds are treated with a hypochlorite solution at pH 10.5, thus forming N-chloro derivatives. The excess reagent is decomposed by an acidified phenol solution, and iodine is liberated from potassium iodide by reaction with the chloramine. The shortcomings of this procedure are the instability of the yellow iodine color, and the narrow range of adherence to Beer's law. These problems were solved by dilution of the yellow color with ethanol. Figure 1 shows the effect of various con-

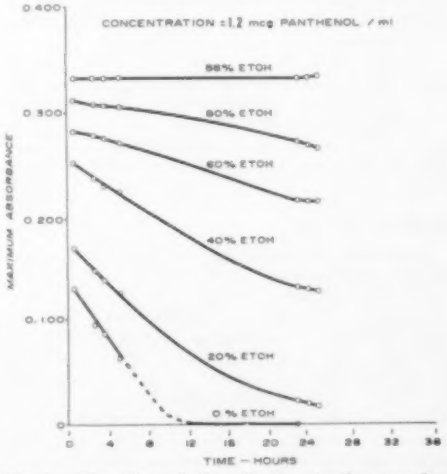

Fig. 1.—The effect of ethanol concentration on color stability and intensity.

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[1] Fortespan Spansule capsules, Smith Kline & French Laboratories.

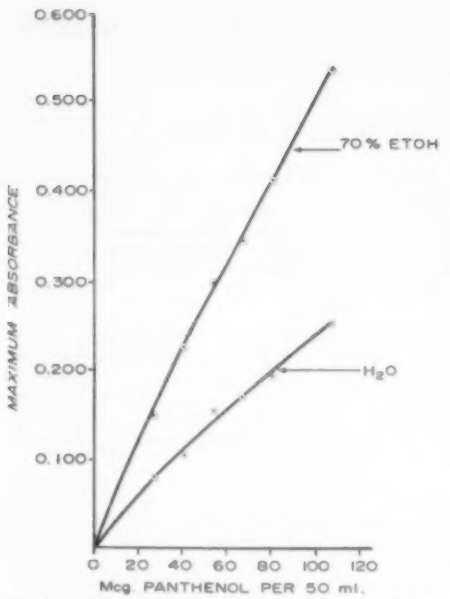

Fig. 2.—Illustration of improved adherence to Beer's law using aqueous ethanol.

centrations of ethanol on color stability and intensity, and Fig. 2 illustrates the improved adherence to Beer's law using aqueous ethanol.

Isopropyl alcohol and tertiary butyl alcohol were used in place of ethanol, but the overall results did not compare favorably with those obtained when ethanol was used as the diluent. Although dilution with isopropyl alcohol increased the color intensity by 25%, considerable variation in results occurred unless the absorbance was determined within thirty minutes. Tertiary butyl alcohol showed no promise at all. Dimethylformamide and 2-methoxyethanol were also tried as diluents, but very high reagent blanks resulted. No attempt was made to purify these solvents.

Schmall and Wollish (*loc. cit.*) used sodium hydroxide to hydrolyze the panthenol and sulfuric acid to neutralize the excess base. The resultant salt precipitated when ethanol was added. No precipitate was encountered when lithium hydroxide and hydrochloric acid were substituted in their places.

Proposed Method

Apparatus.—Chromatographic glass tube of about 19 mm. diameter and 20–25 cm. in length, fitted with a stopcock at the bottom.

Reagents.—Dowex 50W-X4 (H+ form), 100–200 mesh, Baker analyzed reagent. Amberlite CG-400 (Cl-form) type 1, 100–200 mesh. Florisil, 60–100 mesh. Lithium hydroxide 2 *M*, hydrochloric acid 1 *M* and 0.1 *M*. Borate buffer, 0.05 *M*, dissolve 3.1 Gm. of reagent grade boric acid and 3.7 Gm. of potassium chloride in about 900 ml. of water; adjust pH to 10.5 with 2 *N* sodium hydroxide and dilute to 1 L. Chlorinating solution, dilute a 5.25% sodium hypochlorite solution (Clorox) 1 : 30 with 0.05 borate buffer; prepare this solution daily and protect from light. Acidified phenol solution, 0.5% phenol in 0.1 *M* hydrochloric acid. Potassium iodide solution, 1% aqueous; prepare this solution weekly and protect from light. Alcohol, dehydrated (absolute ethyl alcohol). Phenolphthalein indicator, 1% in 95% ethanol. Standards, crystalline *dl*-panthenol dried for four hours at 56° *in vacuo*; U. S. P. reference *d*-calcium pantothenate dried for three hours at 105°.

Preparation of Chromatographic Columns.—The resins are soaked in water for several hours and slurried until most of the fines have been removed. A small plug of glass wool is placed at the bottom of an empty chromatographic tube. A double-bed column is prepared to consist of a 5-cm. height of Dowex 50W-X4 on top of a 5-cm. height of Amberlite CG-400, the two resins being separated by a thin layer of glass wool. Prewashing the "packed" column with 1 *M* hydrochloric acid alleviated low recoveries and column-to-column variations which occurred with some lots of resins. The entire column is then washed thoroughly with 50–75 ml. of distilled water, or more if the hydrochloric acid prewash is found to be necessary. Where pantothenates are to be determined, the same height of Florisil is substituted for the Amberlite.

PROCEDURE

An aqueous solution of the sample is prepared. Solid preparations may be dissolved by homogenizing in a Waring Blendor. An aliquot, preferably 10–15 ml., equivalent to 0.6–1.0 mg. of panthenol or pantothenates, is applied to the column. The liquid is allowed to flow through the column at a rate of 3–4 ml. per minute and is collected in a 100-ml. volumetric flask (or in a 50-ml. volumetric flask when 0.3–0.5 mg. is applied in 10 ml. to the column). Significant losses occurred when slower flow rates were used. When approximately 1 ml. of liquid remains above the bed, the column is eluted with distilled water until the volume of liquid in the flask reaches 80–85 ml. (or 40–45 ml. if 50 ml. is the final volume). Five milliliters of 2 *M* lithium hydroxide are added to the flask which is then heated on a steam bath for forty-five to sixty minutes to insure complete cleavage. The solution is cooled to room temperature and diluted to the mark with water.

A 10-ml. aliquot is pipetted into a 50-ml. volumetric flask and one drop of phenolphthalein indicator is added, followed by the addition of about 1 ml. of 1 *M* hydrochloric acid. Then, the solution is titrated to a colorless end point with 0.1 *M* hydrochloric acid.

One milliliter of chlorinating solution is added to the neutralized aliquot. Care should be taken to deliver the reagent directly into the solution without getting any on the neck of the flask. The solution is swirled, stoppered, and allowed to stand for fifteen minutes. Then 1 ml. of acidified phenol reagent is added, rinsing down the neck of the flask. Again the solution is swirled, stoppered, and allowed to stand for five minutes. Finally, 1 ml. of potassium iodide reagent is added. Immediately, the sample is diluted to the mark with ethanol and mixed well. Its absorbance is determined at 358 mμ on a suitable spectrophotometer.

A reagent blank, consisting of 10 ml. of water and

TABLE I.—COMPARISON OF RESULTS BY CHEMICAL AND MICROBIOLOGICAL METHODS

Sample	Constituents	Assay, mg./Capsule: Microbiological	Assay, mg./Capsule: Chemical
		Panthenol	
1	Vitamins C, B complex	12.1	11.8
2	Vitamins C, B complex	12.0	11.5
3	Vitamins C, B complex	12.0	11.1
4	Vitamins C, B complex	12.1	12.4
5	Vitamins C, B complex	12.3	12.3
6	Vitamins C, B complex	11.7	11.7
7	Gelatin capsules, vitamins A, D, C, B complex	12.1	11.8
		Calcium Pantothenate	
8	Gelatin capsules, vitamins A, D, C, B complex	8.0	8.4
9	Gelatin capsules, vitamins A, D, C, B complex, minerals	27.5	28.6

each of the reagents added at the specified time intervals, is run with the sample. No column blanks need be run since they were found to differ from the reagent blanks by a very insignificant amount.

When new batches of resins are used, standard solutions of panthenol or pantothenates must be put through the entire procedure. A standard factor is determined by dividing the concentration of the solution by its net absorbance. A typical value is 4.10 mcg./ml./absorbance unit at 358 mμ. In the calculation of results, the samples are compared to the standards by applying the factor just mentioned. In the range studied, the retention by the resins is about 5% of the amount of either standard added.

All solutions were read on a Beckman model DU spectrophotometer in a 1-cm. cell, at a wavelength of 358 mμ using air as a reference. Absorption curves of the color reaction show the maximum absorption to be at 358 mμ. From Fig. 1 it may be seen that delays up to three hours will not significantly change the absorbance.

RESULTS

The results obtained by this chlorinating procedure indicate excellent recoveries from several multivitamin preparations, and are in good agreement with those obtained by accepted microbiological methods. The method of György (3) was used to determine panthenol, and that of Skeggs and Wright (4) to determine calcium pantothenate. A comparison of these results is shown in Table I. A standard deviation (s) of 0.2 was obtained using the formula

$$s = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$$

Where x = the individual value, $\bar{x}$ = the mean value, and n = the numbers of values.

DISCUSSION

The most likely source of error in this reaction is in overtitration of the sample with hydrochloric acid, in which case high results are obtained. It should also be mentioned that hypochlorous acid does not react with the panthenol molecule itself. For this reason it was hydrolyzed to β-alanol before color development.

Application of this color reaction to other N-H bearing compounds proved successful, as in the work of Ellis and Hetzel (*loc. cit.*). Excellent results were obtained in our laboratory when determining aqueous solutions of several primary and secondary amines, as well as a substituted imidazole and some carbamates.

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Determination of Antihistamines in Pharmaceutical Products by Nonaqueous Titration

By E. G. CLAIR† and L. G. CHATTEN‡

A simple method is described for the determination of antihistamines in commercially produced tablets and capsules. It consists of extraction of the antihistamine from the pharmaceutical product with chloroform and visual titration of the extract with perchloric acid titrant, after the addition of acetic acid or acetonitrile and a suitable indicator. Most excipients are insoluble in chloroform while still others are not titrated under the conditions of the analysis. The method is rapid and applicable to all but a few of the products tested.

SEVERAL WORKERS have attempted to develop a general procedure for the determination of antihistamines. Bandelin, *et al.* (1), analyzed ten antihistamines colorimetrically, using ammonium reineckate reagent and applied the method with success to five commercial samples of the tablets and capsules as well as to other pharmaceutical products. Blaug and Zopf (2) determined ten pure antihistamine salts using ion exchange and utilized this method successfully in the analysis of tripelennamine hydrochloride in various pharmaceuticals. Rasmussen, *et al.* (3), prepared the picrates of twenty antihistamines and determined their equivalent weights by potentiometric titration in 1 to 1 glacial acetic acid chloroform solution, although this method was not recommended for the quantitative determination of these compounds in commercial products. Other methods have been published for the analysis of antihistamines but for the most part these apply to a single compound (4), or to a limited group of compounds, such as the König reaction (5), specific for compounds with a 2-pyridyl group or the method of Heim (6), which is applied to antihistamines containing an ether group.

Spectrophotometric procedures have been most widely adopted as official methods for determining antihistamines in pharmaceutical preparations, while nonaqueous titrations in glacial acetic acid have been chosen generally for the analysis of the pure compounds (7, 8). Kleckner and Osol (9) determined eight antihistamines by nonaqueous titration in glacial acetic acid using perchloric acid titrant. They found the method to be superior to titration in aqueous or aqueous-alcohol solutions and to spectrophotometry, but no reference was made to the analyses of commercial products by their technique.

The purpose of the present investigation was to develop a visual nonaqueous titrimetric method for the determination of antihistamines occurring in tablets and capsules and to determine the extent of interference caused by excipients in this type of analysis.

EXPERIMENTAL

Materials and Reagents.—Pure crystalline antihistamine salts, as supplied by the manufacturers, were dried and assayed according to U. S. P. (7), N. F. (8), or by the manufacturer's procedure where the drugs were nonofficial; chloroform, reagent grade; glacial acetic acid, reagent grade; dioxane, Eastman white label; acetonitrile, reagent grade, treated with ion exchange resin Amberlite IRC-50 (10); perchloric acid 0.05 *N* in dioxane, standardized against oven-dried potassium biphthalate; mercuric acetate solution, 6% in glacial acetic acid; crystal violet, 0.5% in glacial acetic acid; methyl red, 0.1% in methanol. A Fisher titrimeter with standard glass and sleeve type calomel electrodes was used for all potentiometric titrations.

Procedure.—A sample of powdered tablet or capsule material, calculated to contain 0.15 to 0.25 meq. of antihistamine salt was accurately weighed into a 125-ml. Erlenmeyer flask, 15 ml. of chloroform was added, and the mixture was stirred electromagnetically for fifteen minutes. The solution was passed through a sintered-glass or porcelain filter of medium porosity under suction and the flask and filter were washed with two additional 5-ml. portions of chloroform. For potentiometric titrations, the filtrate was transferred to a 150-ml. beaker, the filter flask was rinsed with 25 ml. of glacial acetic acid or acetonitrile, depending on the solvent system chosen, and the rinsings were added to the filtrate. Visual titrations were performed in the filter flask. In titrating hydrochloride salts, 1 ml. of 6% mercuric acetate solution was added according to the procedure of Pifer and Wollish (11). A blank determination of the solvent system was subtracted from the value obtained for each

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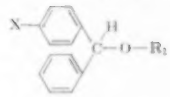
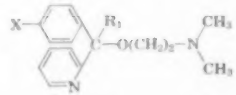
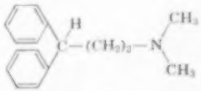
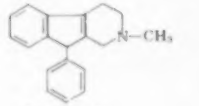
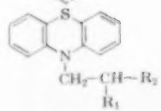
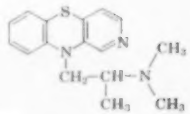
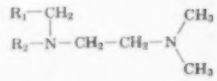
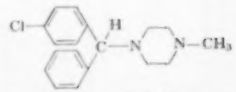
TABLE 1.—RESULTS OF ANALYSIS OF PURE ANTIHISTAMINE SALTS

Group	No.		Recovery, %	Standard Deviation	Gm. Equiv. $HClO_4$/Mole	Substituents X	Substituents R_1	Nucleus of Free Base
A	1	Bromodiphenhydramine HCl	99.5	0.21[a]	1[a, b]	Br	$-CH_2-CH_2-N(CH_3)_2$	
	2	Diphenhydramine HCl	99.7	0.21[a]	1[a, b]	H	"	
	3	Dimenhydrinate	100.2	0.28[a]	1[a, b]	H	"	
	4	Piperinhydrinate	100.2	0.69[a]	1[a, b]	H		
B	5	Doxylamine succinate	97.6	0.23[a]	2[a, b]	H	CH_3	
	6	Carbinoxamine maleate	95.5	0.61[a]	2[a, b]	Cl	H	
C	7	Parabromdylamine maleate	100.1	0.31[a]	2[a, b]	Br		
	8	Chlorpheniramine maleate	98.2	0.46[a]	2[a, b]	Cl		
	9	Pheniramine maleate	98.7	0.51[a]	2[a, b]	H		
D	10	Phenindamine tartrate	...	...	1[b]			
						R_1	R_2	
E	11	Promethazine HCl	99.1	0.48[a]	1[a, b]	CH_3	$-N(CH_3)_2$	
	12	Pyrathiazine HCl	99.0	0.59[b]	1[a, b]	H	N-Pyrrolidine	
F	13	Isothipendyl HCl	99.7	0.52[b]	1[b]			
						Phenyl	2-Pyridyl	
G	14	Tripelennamine HCl	99.4	0.19[a]	2[a]	4-Methoxyphenyl	2-Pyridyl	
	15	Pyrilamine maleate	98.7	0.40[a]	2[a]	2-Thenyl	2-Pyridyl	
	16	Methapyrilene HCl	99.7	0.47[a]	2[a]; 1[b]	4-Methoxyphenyl	2-Pyrimidine	
	17	Thonzylamine HCl	99.7	0.24[b]	2[a]; 1[b]			
H	18	Chlorcyclizine HCl	99.7	0.43[b]	2[a]; 1[b]			

[a, b] Designate the solvent systems used.

sample. The solvent consisting of 25 ml. of chloroform, 25 ml. of glacial acetic acid, and 2 drops of crystal violet indicator is referred to as system a, and the solvent consisting of 25 ml. of chloroform, 25 ml. of acetonitrile, and 2 drops of methyl red indicator is referred to as system b.

RESULTS AND DISCUSSION

In addition to systems a and b, titrations were also carried out in pure chloroform and pure glacial acetic acid. Only the data obtained using the mixed solvent systems are reported in Table I.

In a number of instances a good visual end point was obtained with crystal violet indicator, using 50 ml. of chloroform as solvent. Potentiometric titrations were erratic in this system, however, particularly in the early stages of the titration and, in some instances, the perchlorate salts formed were insoluble in chloroform and their precipitation from solution interfered with the functioning of the electrodes. Methapyrilene hydrochloride represents an extreme case. During the titration of that material, a gummy precipitate formed on the electrodes and rendered the system completely inoperative. The addition of an equal volume of glacial acetic acid or acetonitrile to the chloroform extract produced a smooth titration curve.

While satisfactory titrations for many of the compounds were obtained in glacial acetic acid alone, this solvent was discarded as being impractical for the analyses of pharmaceuticals due to interference caused by a number of excipients, which are extracted along with the active ingredients.

All antihistamine salts were titrated in both systems a and b, and that solvent system was chosen which gave the sharpest change of color at the end point, i.e., where the visual end point coincided with the steepest portion of the potentiometric curve.

Table I contains a list of 18 antihistamine salts which were titrated successfully. These compounds have been divided into eight groups according to molecular structure. The values under per cent recovery are the mean of five determinations; the standard deviation is given for each value. The superscript appended to the standard deviation value indicates, in each instance, the solvent indicator system which gave the sharpest end point. The next column in this table gives the number of equivalents of perchloric acid consumed per mole of antihistamine salt in both systems a and b, and the last column shows the nucleus and substituen ts of the free base for each compound.

Group A compounds are ethers, all with a related molecular structure, as shown in Table I. The four members contain a single, strongly basic nitrogen atom which titrates to a sharp end point in both systems a and b, as shown by Fig. 1, where 1a, 1b, 2a, and 2b are the potentiometric curves of the 8-chlorotheophyllinate and the hydrochloride salts of diphenhydramine. The much smaller $\Delta E/\Delta V$ value at the end point for the hydrochloride salt as compared with the 8-chlorotheophyllinate in system b is due, apparently, to the necessary addition of 1 ml. of mercuric acetate solution to the hydrochloride salt. This assumption seems to be borne out by the fact that the $\Delta E/\Delta V$ values in system a for both salts are of the same order and comparable to

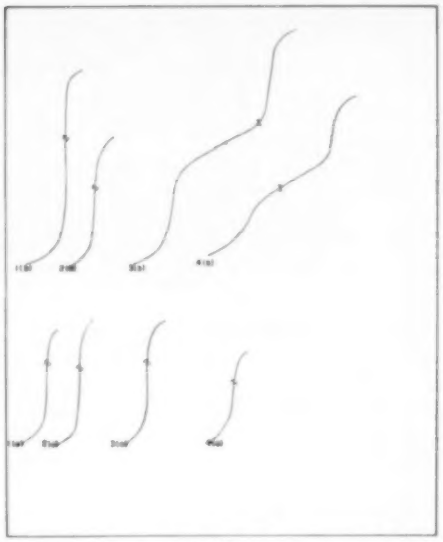


Fig. 1.—Titration curves for compounds from groups A, B, and C. 1. Dimenhydrinate; 2. diphenhydramine hydrochloride; 3. doxylamine succinate; 4. parabromdylamine maleate. (*a*) and (*b*) designate solvent systems used, ~ indicates the visual end point.

the value for the hydrochloride salt in system b. Since the acetate is a weaker base than the 8-chlorotheophyllinate, the rate of change in potential at the end point is less. The decreased $\Delta E/\Delta V$ values caused by the presence of acetic acid in the titrating solution does not impair the visual end point appreciably for these compounds in either systems a or b.

In the two members of group B, a 2-pyridyl ring replaces a benzene ring in the benzhydryl portion of the molecule. The pyridine nitrogen in these compounds is considerably less basic than the alkylamine nitrogen and, in system b, the two nitrogen atoms are differentiated potentiometrically. The indicator is not sharp in system b since, as shown in Fig. 1, 3b, it occurs on a flat portion of the titration curve, while in system a the two nitrogens are leveled and the visual end point is sharp.

Group C is composed of dialkylamino compounds. All members of this group give titration curves which are very similar to that of parabromdylamine in Fig. 1, 4a and 4b, and to those of the ethers in group B, as seen in Fig. 1.

Since the maleate of carbinoxamine differs from that of chlorpheniramine by the presence of an ether oxygen in the former compound, it might be expected that the electronegativity of this moiety would influence the titratability of carbinoxamine. However, this was not demonstrated experimentally.

Phenindamine tartrate does not appear to belong to any group, and therefore was placed alone in Table I. It might be considered to correspond to a dialkylaminoalkyl compound in which the side chain has undergone ring closure. Differences were thought to be too significant, however, for in-

clusion in group C. This compound has a low solubility in chloroform, but is readily solubilized by the addition of phenol, as previously reported by one of us (12).

The compounds of groups E and F are dialkylaminoethylamines. As might be expected, since the nitrogen of phenothiazine is not basic, those of group E consumed only one equivalent of perchloric acid per mole in systems a and b and they give sharp end points in both instances. Isothipendyl hydrochloride has the structure of promethazine hydrochloride in which a 2-pyridyl ring is substituted for one of the fused benzene rings in the phenothiazine nucleus. The additional nitrogen in this compound interferes with the end point in system a, while in system b only the alkylamine nitrogen is sufficiently basic to titrate and the resulting end point is sharp.

The group G compounds contain a substituted ethylenediamine moiety but have no central polyaromatic nucleus as do the compounds in groups E and F. The first three members of this series have similar substitutions on the ethylenediamine nitrogens; all have one nitrogen atom substituted with a pyridine ring and a second aromatic group which may be a benzyl, a thenyl, or a substituted benzyl or thenyl group. In system a the two basic nitrogen atoms in all three compounds are leveled and each compound consumes two equivalents of perchloric acid to give a sharp end point. Titration of these substances in system b resulted in a poor visual end point in each instance since the color change of the indicator failed to coincide with either potentiometric inflection point.

The pyridine ring found in the other members of this group is replaced in thonzylamine hydrochloride by pyrimidine. Only one nitrogen atom of thonzylamine was sufficiently basic to titrate in system b and a sharp end point was obtained. In system a, the basicity of a weaker nitrogen atom was enhanced, although not to the level of the more basic nitrogen, and a differential titration curve resulted The visual end point was not as sharp in this system as in system b. Thonzylamine hydrochloride is the only antihistamine salt encountered in which two nitrogen atoms are differentially titrated in system a, the weaker nitrogen was sufficiently basic to be titrated with perchloric acid but was too weakly basic to be leveled by acetic acid. Figure 2 shows the titration curves of tripelennamine hydrochloride and thonzylamine hydrochloride in both systems a and b.

Chlorcyclizine hydrochloride has been placed in group H, since it does not fit clearly into any of the other groups shown in Table I. The piperazine group of chlorcyclizine may be compared with the substituted ethylenediamine chain found in compounds of group G. It has been shown that in ethylenediamine (13) and in 1,4-disubstituted piperazines (14), the inductive effect of the two nitrogens on each other will mutually hinder their basic action. If one of the amine nitrogens is protonated, its inductive effect on the other amino nitrogen will be greatly enhanced and will inhibit the ease of proton acceptance of the less basic nitrogen atom. This, in part, explains the lack of basic properties in one of the nitrogen atoms of the ethylenediamine chain in group G compounds and the difference in basicity of the two nitrogen atoms in the piperazine

Fig. 2.—Titration curves for compounds from group G. 1. Tripelennamine hydrochloride; 2. thonzylamine hydrochloride. (*a*) and (*b*) designate the solvent systems used, ~ indicates the visual end point.

ring of chlorcyclizine hydrochloride. In system b only one nitrogen of chlorcyclizine titrates to give a sharp end point. In system a, both nitrogens are titrated, showing that the inductive effect is not as pronounced in the piperazine ring as in ethylenediamine. The end point in this system is not as sharp, however, as it is in system b.

It should be pointed out that the proposed procedure was applied also to the following antihistamines, but because of their poor solubility in chloroform the technique was considered to be unsuitable; chlorothen citrate, antazoline hydrochloride, N-benzyl-N′,N′-dimethyl-N-phenylethylenediamine maleate, methaphenilene hydrochloride, phenyltoloxamine dihydrogen citrate, and thenalidine tartrate. The addition of phenol to the chloroform did enhance the solubility in some instances, but the resulting indicator end point was unsatisfactory.

Thenyldiamine hydrochloride is soluble in chloroform, but upon addition of perchloric acid, a precipitate formed which interfered with the functioning of the electrodes. Even the addition of an equal part of glacial acetic acid did not prevent this phenomenon. It is interesting to note that the visual end point was satisfactory, but quantitative data on this compound is not reported herein because the visual end point could not be confirmed by potentiometric titration.

Table II shows the recoveries of a number of antihistamines from pharmaceutical products. In each instance the nonaqueous method found to be most suitable, as indicated in Table I, was used. The determinations were performed in triplicate and the mean value has been reported. The values shown under U. S. P. method are the mean of duplicate determinations of the same sample. The agree-

Table II.—Results of Analyses of Pharmaceuticals

No.	Compound	Total Wt., mg./Tablet	Declared, mg./Tablet	Found mg./Tablet: Nonaqueous Method	Found mg./Tablet: U. S. P. Method
1	Bromodiphenhydramine hydrochloride	250.0	25.0[a]	24.4	25.6[b]
2	Diphenhydramine hydrochloride	141.0	25.0[a]	25.1	25.5
3	Dimenhydrinate	181.7	50.0	50.2	50.8
4	Doxylamine succinate	178.3	25.0	23.5	25.6
5	Methapyrilene hydrochloride	212.0	25.0	25.8	25.8
6	Pyrilamine maleate	120.0	50.0	47.0	47.6
7	Pyrilamine maleate	131.0	50.0	46.5	46.1
8	Tripelennamine hydrochloride	199.2	50.0	44.9	44.6
9	Thonzylamine hydrochloride	99.5	25.0	23.2	23.3
10	Chlorcyclizine hydrochloride	148.0	50.0	49.6	49.8
11	Chlorcyclizine hydrochloride	186.0	50.0	51.0	50.8
12	Phenindamine tartrate	268.6	25.0	24.6	26.0
13	Isothipendyl hydrochloride	126.6	4.0	4.0	4.1[b]
14	Chlorpheniramine maleate	195.0	4.0	3.6	3.9
15	Piperinhydrinate	105.4	3.0	3.0	...[c]

[a] Capsules, all other products in the form of tablets. [b] Manufacturer's method of analysis. [c] Insufficient sample for U. S. P.

ment between the two sets of values shows that the nonaqueous titration method is applicable to tablets and capsules ranging from 3 mg., in which the active constituent comprises less than 3% of the total weight of the tablet, to 50 mg., where the active constituent comprises more than 40% of the total weight.

In the instance of promethazine hydrochloride, the rather low solubility of this salt in chloroform results in a recovery from commercial tablets which is low and has poor reproducibility, consequently no results are reported for this preparation. For tablets of parabromdylamine maleate, something in the formulation inhibited extraction of the active component, despite its ready solubility in chloroform.

To ascertain the maximum amount of interference which could be caused by the common excipients known to be present in tablets and capsules, various solvents were saturated with these materials by adding an excess amount to 50 ml. of solvent and stirring the solution for fifteen to twenty minutes. The undissolved material was filtered off and the clear saturated solution was titrated with perchloric acid. Table III shows the amount of perchloric acid (0.05 *N*) required to titrate 50 ml. each of chloroform and acetic acid to a crystal violet end point when saturated with the various excipients. Examination of the table shows that less than one-third of the materials listed increased the titratable blank in the chloroform extraction and, in only a few instances, was the amount of increase significant. The most serious interference was caused by polyethylene glycol 4000, polyvinyl pyrrolidone, and magnesium hydroxide. Polyvinyl pyrrolidone occurs in the chlorcyclizine hydrochloride sample No. 11 in Table II to the extent of 8–9% of the weight of the active ingredient. The analysis by nonaqueous titration is in close agreement with analysis by the official procedure, although the result is slightly high by both methods. The other two excipient materials showing the greatest amount of interference in the chloroform extract did not occur in any of the pharmaceuticals examined.

One of the excipients which occurs commonly in tablet formulations is magnesium stearate. This material has been cited frequently as a major cause of interference in the determination of pharmaceutical bases by nonaqueous titration methods. For

Table III.—Extent of Interference Caused by Commonly Used Excipients

Excipient	ml. $HClO_4$ (0.5 *N*): $CHCl_3$[a]	ml. $HClO_4$ (0.5 *N*): CH_3COOH[b]
Acacia	0.04	0.15
Beeswax	0.00	0.00
Calcium carbonate	0.00	0.13
Calcium phosphate dibasic	0.04	2.00
Calcium phosphate tribasic	0.00	0.58
Calcium stearate	0.06	2.00+
Calcium sulfate	0.00	0.70
Carnauba wax	0.00	2.00+
Cetyl alcohol	0.02	0.00
Gelatin	0.00	2.00+
Lactose	0.00	0.02
Magnesium carbonate	0.00	2.00+
Magnesium hydroxide	1.06	2.00+
Magnesium stearate	0.14	2.00+
Magnesium sulfate	0.00	0.08
Methylcellulose	0.00	0.34
Polyethylene glycol 4000	2.00+	0.06
Polyvinyl pyrrolidone	2.00+	2.00+
Sodium alginate	0.00	0.98
Sodium benzoate	0.00	2.00+
Sodium carboxymethylcellulose	0.06	2.00+
Sodium silicate	0.00	2.00+
Sodium sulfate	0.00	1.18
Starch (potato)	0.00	0.06
Sugar (powdered)	0.00	0.00
Talcum	0.14	0.50
Tragacanth	0.00	0.38

[a] A 50-ml. quantity of chloroform saturated with excipient. [b] A 50-ml. quantity of glacial acetic acid saturated with excipient.

this reason it was examined closely to determine the extent of interference it causes with this method of analysis. In one tablet formulation, magnesium stearate occurs in the ratio of 1 equivalent to 3 equivalents of active ingredient. When the tablet was dissolved in acetic acid and titrated, the value obtained was 25% higher than the declared value. When it was extracted with chloroform and filtered before titrating, the value obtained was 1.7% lower than the declared value. To further investigate the interference caused by magnesium stearate, the components of the tablet formulation were mixed together in the same proportion as they occur in the commercially prepared tablet and appropriate aliquots were extracted with chloroform. The ex

tract was filtered, washed, and determined in the solvent system found to be most suitable for the titration of the pure antihistamine salt; in no instance was there any evidence of interference by magnesium stearate or any of the other excipients contained in the formulation. To check that compensating errors were not the cause of the favorable results, duplicate samples of the active ingredients were weighed into beakers; to one beaker was added an amount of magnesium stearate proportionate to that occurring in the tablet formulation, both samples were then extracted with chloroform, filtered, and titrated. The results obtained are shown in Table IV.

TABLE IV.—ANALYSIS OF SYNTHETIC TABLET FORMULATION

Added, mg.		Recovered, %
Active Ingredient	Magnesium Stearate	Active Ingredient
71.00	0	100.25
62.95	0	99.70
62.00	14.45	99.10
66.90	12.20	100.10

Since the formulation investigated contains an extremely large amount of magnesium stearate, it is very unlikely that magnesium stearate will cause significant interference in most tablets, where its ratio to that of the active ingredient is much less.

While most excipient materials contribute little or no positive error to this method of assay, the active ingredient, although extremely soluble in chloroform, was incompletely extracted from one tablet which had a high content of dibasic calcium phosphate. Since dibasic calcium phosphate is only slightly soluble in chloroform, it was assumed that the low recovery of active ingredient was caused either by its occlusion within the finely ground particles of dibasic calcium phosphate or by the formation of an insoluble complex during the tableting process.

It has long been the general feeling that the effect which tablet excipients might exert on the quantitative analysis of this pharmaceutical form by nonaqueous titrimetry is probably so great as to limit its usefulness to the assay of the basic materials alone or, at best, to those manufacturers where the control laboratory might be able to exert its influence in the formulation. In our opinion, such a situation is far from actual fact.

While it is true that we have encountered several instances of interference by excipients, when one considers the wide application which has been made of nonaqueous titrimetry over the years, such occasions constitute only a small percentage of the products investigated. At present, investigations are proceeding on the influence of tablet excipients in a number of solvent systems and the results will appear in a subsequent publication.

SUMMARY

1. A nonaqueous titrimetric procedure has been developed for 17 antihistamines which is extremely simple and yet yields results comparable in precision and accuracy with methods which are more time consuming or require more elaborate instrumentation.

2. Of the 7 antihistamines which do not lend themselves to this technique, none are official in the U. S. P. XVI.

3. Investigation of the effects of tablet excipients on the method reveals that in only a very few instances is there any cause for concern.

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Calcium sulfate	0.00	0.70
Carnauba wax	0.00	2.00+
Cetyl alcohol	0.02	0.00
Gelatin	0.00	2.00+
Lactose	0.00	0.02
Magnesium carbonate	0.00	2.00+
Magnesium hydroxide	1.06	2.00+
Magnesium stearate	0.14	2.00+
Magnesium sulfate	0.00	0.08
Methylcellulose	0.00	0.34
Polyethylene glycol 4000	2.00+	0.06
Polyvinyl pyrrolidone	2.00+	2.00+
Sodium alginate	0.00	0.98
Sodium benzoate	0.00	2.00+
Sodium carboxymethylcellulose	0.06	2.00+
Sodium silicate	0.00	2.00+
Sodium sulfate	0.00	1.18
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Synthesis of Some Quaternary Pilocarpine Salts

By NORBERT J. WOJCIECHOWSKI and BERNARD ECANOW

Pilocarpine is classed as a cholinergic drug. Unlike the choline analogs and derivatives, pilocarpine is not a quaternary ammonium but a tertiary amine derivative. In order to study the pharmacological activity of the quaternary salts of pilocarpine, a series of such salts were synthesized. Also prepared were a series of new picrate salts of the pilocarpine derivatives. The optical activity and the specific rotations of the new derivatives are reported. The infrared spectra were determined also.

PILOCARPINE is classed as a cholinergic drug. Unlike the choline analogs and derivatives, pilocarpine is not a quaternary ammonium but a tertiary amine derivative. In order to study the pharmacological activity of the quaternary salts of pilocarpine, a series of such salts were synthesized. Alkylation experiments on pilocarpine were previously reported (1–3), using lower members of the alkyl halides.

The basic procedure followed was the addition of an excess of the alkylating agent to pilocarpine base. With the lower members of the series, i.e., methyl and ethyl iodides, the reaction was exothermic, and almost spontaneous. In ten to twenty minutes at room temperature, an oily layer of product was deposited.

With increasing molecular weight of the alkylating agent, the reaction rate became slower. The application of heat to the reaction flask increased the reaction rate but also increased the formation of a discolored product. Less discoloration resulted when the reactants were refluxed in a solvent, either alcohol, chloroform, or nitrobenzene, than when the mixture of reactants alone were warmed. The yields, by the variously modified procedures, ranged from 76.1 to 88.8% of the theoretical yields (see Table I).

The melting points of these compounds decrease with an increase in the molecular weight, and/or more specifically, decrease with an increase in the length of the alkyl substituent introduced into the molecule (Table I).

The preparation of picrate derivatives of these quaternary compounds for the most part are readily obtained. They are discussed in the experimental section. Their idiosyncratic behavior did not result in one general method of preparation for all members of the series, but several methods for the isolation of the picrates was necessary. This is presumably due to the increasing solubility of these picrates in alcohol as you go up the series. The picrates were prepared so that additional means of identification for these quaternary pilocarpine compounds was available. In the case of the hexyl and octyl members, the picrates were the only solid forms of these compounds that were obtained. The physical characteristics of the picrates are tabulated (Table II).

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EXPERIMENTAL

Preparation of *n*-Butyl Pilocarpinium Iodide.—Five and one-half milliliters of 1-iodobutane was added, with efficient stirring, to the pilocarpine base obtained from the neutralization and chloroform extraction of 6.25 Gm. of pilocarpine hydrochloride. Absolute ethanol, 35 ml., was also added and the mixture refluxed for six hours. The temperature was almost a constant 79° throughout the refluxing period.

The reaction mixture remained at room temperature for twelve hours and was then placed in the freezing compartment of a refrigerator. Within one hour a gel-like portion formed and a clear supernatant portion (excess alkylating agent) separated and could be poured off. The gel was dissolved in 25 ml. of absolute methanol, treated with Norit, and filtered. Anhydrous ether was added to the methanol solution until the latter became turbid. The solution was then placed in a refrigerator freezing compartment. After one hour there was a precipitation of a yellowish oil. The oil was separated and redissolved in 25 ml. absolute methanol. Five milliliters of the methanol solution was poured into a glass crucible and the solution evaporated to dryness on a hot plate set to low heat. The crucible was then placed into an evacuated desiccator. To the remaining 20 ml. of methanol solution was added enough ether to reach the point of turbidity. This solution was then returned to the refrigerator. After two days, the crucible was removed from the desiccator. A yellowish-white powder was scraped from its sides. This powder was used to seed the oil which had separated from the methanol-ether solution in the refrigerator. After about seven hours a white crystalline salt was collected which was recrystallized from alcohol.

Isoamyl Pilocarpinium Iodide.—Prepared essentially by the same method used for the *n*-butyl derivative. Product has a white crystalline structure.

Hexyl Pilocarpinium Iodide and Octyl Pilocarpinium Iodide.—Prepared by the same method used

TABLE I.—QUATERNARY PILOCARPINIUM SALTS

Compound	M.P., °C.	Specific Rotation[a] Concn., Gm.		Nitrogen, % Calcd.	Found	Yield
n-Butyl pilocarpinium iodide	106–108	+57.66	4.006[b]	7.14	7.01	78.8
Isoamyl pilocarpinium iodide	102.5–104	+54.38	4.009[b]	6.89	6.80	76.1
Hexyl pilocarpinium iodide	oil	+44.35	2.029[c]	6.66	6.62	88.8
Octyl pilocarpinium iodide	oil	+41.68	2.327[c]	6.25	6.08	78.0

[a] Temperature, 20°C. [b] Solvent is water. [c] Solvent is ethanol.

TABLE II.—QUATERNARY PILOCARPINIUM PICRATES

Compound	M.P., °C.	Appearance and Crystallization Solvent
Ethyl pilocarpinium picrate	153–154.5	Dark yellow needles (H_2O)
Butyl pilocarpinium picrate	123–125	Orange-yellow needles (H_2O)
Isoamyl pilocarpinium picrate	75.5–77	Dark yellow crystalline powder (EtOH + H_2O)
Hexyl pilocarpinium picrate	85–87	Yellow fine crystalline powder (MeOH + H_2O)
Octyl pilocarpinium picrate	Softens and shrinks 68°; Melts, 75.5–77°	Yellow powder (MeOH + H_2O)

for the *n*-butyl derivative. These two salts were obtained only in the form of oils.

Ethyl Pilocarpinium Picrate, Butyl Pilocarpinium Picrate, Isoamyl Pilocarpinium Picrate, Hexyl Pilocarpinium Picrate, and Octyl Pilocarpinium Picrate.—An alcoholic solution of picric acid was poured into an alcoholic solution of the respective pilocarpinium iodide salt. In the case of the ethyl derivative, the picrate precipitated from solution and could be recrystallized from alcohol. In the case of the other derivatives, water was added to the point of turbidity and the solutions placed in a refrigerator for ten to twelve hours. The precipitated picrates were collected and dried in a vacuum desiccator (twelve to twenty-four hours). The picrate of the octyl derivative had a characteristic melting behavior which was not altered after several recrystallizations.

Optical Activity.—The optical activity of all the compounds synthesized as well as other pilocarpine salts was studied, and all are dextrorotatory. The specific rotation of these compounds is not only a function of the concentration of pilocarpine represented in these compounds but appears to be more basically dependent on the molecular concentration of the asymmetric center present in the solution (Table II).

Infrared Analysis.—Infrared analyses were performed on the hydrochlorides of pilocarpine and isopilocarpine, the quaternary pilocarpinium iodides and quaternary isopilocarpinium iodide, and on pilocarpine. The solids were run as dispersions in potassium bromide (pressed disk technique). The spectra of the oils (hexyl and octyl compounds) and pilocarpine base were obtained on chloroform solutions. The spectra were obtained on a Beckman infrared spectrophotometer, model IR4.

It appears that there is a close analogy between the absorption spectra of pilocarpine, its hydrochloride salt, and the quaternary pilocarpinium iodides. It is difficult to discern any major difference in the spectra due to the effect of quaternization; indeed, the spectra of all compounds, at best, allows one to point out the absorptions due to a 5-membered lactone ring and the elements that may be found in the imidazole ring.

SUMMARY

1. Four new quaternary pilocarpine compounds were prepared: butyl pilocarpinium iodide, isoamyl pilocarpinium iodide, hexyl pilocarpinium iodide, and octyl pilocarpinium iodide.

2. Five new picrates were prepared: ethyl pilocarpinium picrate, butyl pilocarpinium picrate, isoamyl pilocarpinium picrate, hexyl pilocarpinium picrate, and octyl pilocarpinium picrate.

3. The optical activity and the specific rotation of the new quaternary pilocarpine compounds are reported.

4. The infrared spectra of the quaternary pilocarpine compounds were determined.

5. The results of the pharmacologic investigation of the quaternary pilocarpine compounds will be reported in a forthcoming paper.

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Steroid Borates III

Use in Preparation of Triamcinolone 21-Hemisuccinate and Other 21-Monoesters

By LEWIS J. LEESON, JAMES A. LOWERY, GEORGE M. SIEGER, and CHARLES KRIEGER

The syntheses of pure 21-hemi-acid esters of adrenocorticoids possessing the 16α,17α-dihydroxy moiety have previously not been reported. Preparation of these compounds has been dependent upon selective separation of a mixture of 21-mono- and 16,21-diesters. A method for the direct synthesis of pure 21-monoester is described employing the steroid borate complex as a reaction intermediate. This procedure is adaptable as a general synthesis for all 21-monoesters of steroids having the above-mentioned *cis*-diol configuration.

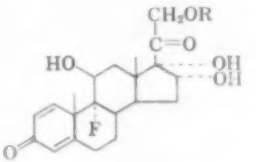
PART II of this series (1) discussed a method of preparing 21-monoesters of 16α,17α-hydroxylated adrenocorticoids. This consists of allowing the steroid to react with an acid anhydride and separating the desired 21-monoester, from the 16,21-diester, by its preferential solubility in aqueous sodium tetraborate solution.

The above procedure, although useful for simple esters, is not applicable to hemi-acid esters, such as the succinate, phthalate, or maleate (1). These latter compounds are important in pharmaceutical formulations since their sodium salts demonstrate a marked increase in water solubility over that of the parent steroid. This report describes a method of preparing such esters by reacting triamcinolone[1] and triamcinolone-like steroids, as the borate complex (2), with a dicarboxylic acid anhydride. To the authors' knowledge, this is the only direct synthesis of these compounds which does not require a selective separation step, such as a chromatographic procedure. Furthermore, this process has been found to be applicable as a general preparative method for triamcinolone 21-monoesters, represented by structure I, and is considered superior to the procedure described previously (1).

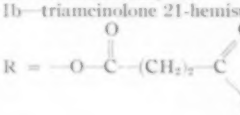
EXPERIMENTAL

Chromatography.—A paper chromatographic system was employed for determining the nature of the products formed in the reaction between triamcinolone and succinic anhydride. The system which gives maximum resolution is butanol/0.05–0.10 *N* ammonium hydroxide using Whatman No. 1 paper. The color was developed with blue tetrazolium spray (3, 4).

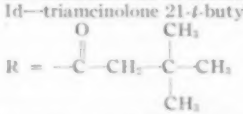
Employing this system the R_f value for triamcinolone was found to be 0.8. The corresponding values for the 21-monohemisuccinate and 16,21-dihemisuccinate esters were 0.4 and 0.1, respectively.



Ia—triamcinolone, R= —H
Ib—triamcinolone 21-hemisuccinate,



Ic—triamcinolone 21-acetate, R = —C(=O)—CH₃
Id—triamcinolone 21-*t*-butylacetate,



Investigation of Reaction Conditions for Hemisuccinate Formation.—*Temperature Study.*—Equimolar amounts of triamcinolone and succinic anhydride were dissolved in pyridine (at a 5–25% steroid concentration) and permitted to react at temperatures of 0, 25, 37, 60, and 100°. At various time intervals a sample was removed, spotted directly onto a paper strip, and chromatographed using the previously described system. The nature of the reaction products was obtained from each chromatogram.

Reactant Ratio Study.—Pyridine solutions were prepared as described above with succinic anhydride and triamcinolone in molar reaction ratios of 3:1, 5:1, and 10:1. These were stored at 100°. At various time intervals samples were removed and chromatographed as in *Temperature Study*. The identity of the products was determined from the chromatogram.

Preparation of Triamcinolone 21-Hemisuccinate (Ib).—One gram of triamcinolone sodium borate (2) was dissolved in 5 ml. of pyridine, and 0.5 Gm. of succinic anhydride added. The solution was permitted to stir overnight and poured into 100 ml. of iced, dilute (2 *N*) hydrochloric acid solution.

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The authors are indebted to Mr. Louis Brancone and coworkers for obtaining the microanalytical data and to Mr. William Fulmor and his staff for their help in interpreting the infrared and ultraviolet spectra of these steroid derivatives.

[1] American Cyanamid Company's trademark for triamcinolone is Aristocort.

The observed precipitate was collected and, when submitted to paper chromatography, showed only one spot with the R_f value of 0.4. Recrystallization from aqueous methanol resulted in a white crystalline solid in approximately 75% yield; m. p. 219–223° (uncor.); E_{239} = 16,000; $[\alpha]_D^{25}$ + 60.5° (CH_3OH). The infrared spectrum was in agreement for the proposed compound.

Anal.—Calcd. for $C_{25}H_{31}FO_8$: C, 60.72; H, 6.32; F, 3.84; neut. equiv., 494. Found: C, 60.93; H, 6.46; F, 4.16; neut. equiv., 503.

Preparation of Triamcinolone 21-Acetate (Ic).—Triamcinolone sodium borate, 1.25 Gm., was dissolved in 4 ml. of dimethylformamide and 0.2 ml. of pyridine. Acetic anhydride, 0.4 ml., was added dropwise and the reaction mixture permitted to stir for six to seven hours at room temperature. The solution was poured into 25 ml. of cold 2 *N* hydrochloric acid solution. The observed precipitate was collected and recrystallized from aqueous acetone; m.p. 215–217° (uncor.); E_{237} = 15,800; $[\alpha]_D^{25}$ + 74.5° (CH_3OH). An infrared spectrum indicated this material to be triamcinolone 21-monoacetate.

Anal.—Calcd. for $C_{23}H_{29}FO_9$: C, 63.29; H, 6.70; F, 4.35. Found: C, 63.35; H, 6.71; F, 4.57.

Preparation of Triamcinolone 21-Tertiarybutylacetate (Id).—One-half gram of triamcinolone sodium borate was dissolved in 7 ml. of pyridine, and 1 ml. of *t*-butylacetyl chloride introduced. The solution was mixed for six hours at room temperature, after which it was added to 40 ml. of water. Cold 2 *N* aqueous hydrochloric acid solution was introduced until there was no further precipitation. The solid was collected and recrystallized from acetone-petroleum ether solution resulting in a white crystalline solid; m.p. 221–222° (uncor.); E_{237} = 15,000; $[\alpha]_D^{25}$ + 74.3° (CH_3OH). The infrared spectrum indicated it to be triamcinolone 21-*t*-butylacetate.

Anal.—Calcd. for $C_{27}H_{37}FO_7$: C, 65.83; H, 7.57; F, 3.86. Found: C, 65.93; H, 8.11; F, 3.82.

DISCUSSION

It would be expected that a reaction involving equimolar quantities of triamcinolone (Ia) and succinic anhydride would result in the compound triamcinolone 21-hemisuccinate. Early experiments, however, indicated the product to be a mixture of starting material, and the 21-mono- and 16,21-dihemisuccinate. This conclusion was based on elemental analyses, neutralization equivalent data, and infrared spectra. A 2:1 molar reaction ratio of anhydride to steroid yielded similar results. It was therefore decided to investigate the effect of reaction conditions on the nature of the products to determine whether simple variations in these conditions would result in formation of only 21-monoester. The previously described paper chromatographic system was employed in this study.

Upon chromatography of material obtained from the reaction between triamcinolone and succinic anhydride, three spots were observed on the strips. The first of these (R_f = 0.8) was readily identified by means of a control, as triamcinolone. However, since authentic samples of the esters were unavailable, the identities of the two remaining spots were assigned on the basis of polarity. Therefore, because of their respective polarities, the second and third spots (R_f = 0.4 and 0.1) were identified as the 21-mono- and 16,21-dihemisuccinate esters. It was confirmed that these assignments were correct when pure monoester was prepared and characterized by physical and chemical analysis.

Investigation of Reaction Conditions.—When equimolar quantities of triamcinolone and succinic anhydride were reacted at temperatures from 0 to 100°, samples were removed over varying time periods of from < one minute to seven days. The results, as interpreted from paper chromatograms, indicated that regardless of temperature a 1:1 steroid to anhydride reaction ratio produced a mixture of starting material, monoester, and diester. As the temperature approached 100°, the major components appeared to be starting material and diester.

When succinic anhydride and triamcinolone were reacted in 3:1, 5:1, and 10:1 molar ratios for time periods of from < one minute to seven hours, two new spots were encountered on the paper chromatograms. The first of these had an R_f value of 0.35 and was considered to be the 16-monoester. The second, with the R_f value of <0.1 (approx. 0.06), was interpreted as the 16,17,21-triester. However, these materials, not being obtained in significant amounts, were felt to be of minor interest.

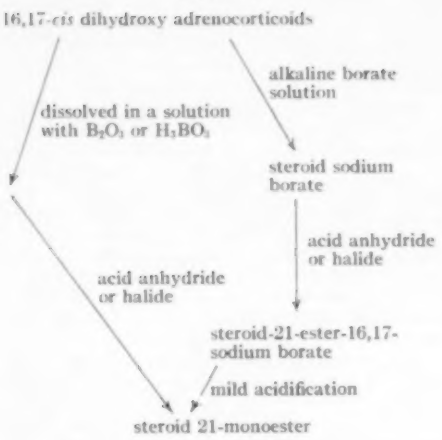
The results of these experiments, interpreted from the paper chromatograms, indicated that after a reaction time of three hours the major component was the diester. It was therefore concluded that pure triamcinolone 21-hemisuccinate could not be prepared directly by using succinic anhydride and triamcinolone regardless of the temperature or molar reaction ratios. As a result, a new procedure for preparing the monoester was investigated.

Reaction Procedure Employing the Steroid Borate Complex.—Since it was recognized that the 16-hydroxyl group of triamcinolone sodium borate was involved in complex formation (2), the possibility that this position would not be available in a reaction between the steroid borate and succinic anhydride was considered. When triamcinolone sodium borate was combined with succinic anhydride at room temperature for twenty-four to seventy-two hours in a 1:1, 1:2, or 1:3 molar ratio, a paper chromatogram of the product showed only one spot (R_f = 0.4). Elemental analyses, neutralization data, and infrared spectra demonstrated the material to be triamcinolone 21-hemisuccinate. This ester was easily purified by recrystallization. Furthermore, this synthetic procedure is readily adaptable as a production process. In addition, it was found that replacing triamcinolone sodium borate with a solution of triamcinolone and boric oxide or boric acid gave the same results.

Since this technique performed so well in the preparation of 21-hemi-acid esters, it was investigated as a general method of preparing 21-monoesters of triamcinolone and triamcinolone-like steroids. To demonstrate this, triamcinolone 21-monoacetate and 21-mono-*t*-butylacetate were prepared and characterized. The general process is summarized in scheme A.

Some additional monoesters made by using scheme A are listed as follows: triamcinolone 21-propionate and butyrate; 1,2-dihydrotriamcinolone 21-acetate,

Scheme A



propionate, butyrate, and *t*-butylacetate; triamcinolone 21-hemiphthalate and hemimaleate; and 1,2-dihydrotriamcinolone 21-hemisuccinate, hemiphthalate, and hemimaleate.

SUMMARY AND CONCLUSIONS

1. The difficulty of preparing triamcinolone 21-monohemisuccinate by reacting succinic anhydride and triamcinolone has been discussed along with a method for studying the course of this reaction via a paper chromatographic technique.

2. A process has been described for preparing triamcinolone 21-monohemisuccinate in good yield by reacting triamcinolone borate with succinic anhydride.

3. The general application of this process for preparing 21-monoesters of 16-hydroxylated adrenocorticoids is discussed.

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Reduction of Tetramine Toxicity by Sedatives and Anticonvulsants

By ELBERT VOSS, A. R. HASKELL†, and L. GARTENBERG

This investigation was undertaken for the purpose of obtaining information which might be applicable to the relief of accidental intoxication with tetramine, a compound which has been used as an antifungal agent and rodent repellent. Screening studies were first made on white rats using seven different compounds possessing sedative and anticonvulsant properties to determine their effectiveness in counteracting the convulsant and lethal effects of tetramine. From these qualitative studies, sodium bromide, sodium phenobarbital, and sodium barbital were found to be most effective and were chosen for further investigation. Each of these three compounds afforded protection against a lethal dose of tetramine when administered to Swiss white male mice in a small percentage of its median lethal dose.

IN A PREVIOUS publication Haskell and Voss (1) reported findings obtained from animal studies using tetramine in the form of a saturated aqueous solution. It was found that tetramine was essentially a central nervous system stimulant with greatest action on the brain stem, and that it induced clonic convulsions which were the immediate cause of death in lethal doses. Tetramine was found to be readily absorbed from mucous membranes in aqueous solution and, in saturated aqueous solution given intraperitoneally to rats, to be destroyed at a rate somewhat less than one-fourth the median lethal dose (LD_{50}) per day. The saturated aqueous solution, when stored at room temperature in nonactinic containers, was found to remain stable for periods up to five months.

Tetramine was reported by Hagen (2) to be approximately five times as toxic as strychnine for mice, the LD_{50} in this animal being 0.1 to 0.2 mg./Kg. wt., and for the albino rat, 0.22 mg./Kg.

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wt. The LD_{50} for other common laboratory animals was reported to be of the same order of magnitude. Platcow (3) confirmed the LD_{50} reported by Hagen for the albino rat.

Tetramine has been found to be an extremely toxic compound for all animal species so far investigated (1, 3, 4). It has been used commercially as an impregnating, stiffening, and antimold agent for fibers used in pillows and upholstery (2), and as a rodent repellent in various solutions used for impregnating seeds employed in reforestation projects (5, 6, 7). Such practical applications would appear to be hazardous in view of the extreme toxicity of tetramine. Adequate measures for counteracting tetramine intoxication have not been reported so far. This investigation was undertaken for the purpose of obtaining information which might be applicable to the relief of accidental intoxication with this compound.

It was first assumed that any agent which would counteract or reduce the toxicity of tetramine would, of necessity, be a chemical antagonist which would inactivate or destroy tetramine in the body, or a pharmacological antagonist which would counteract the central stimulative effects of the drug. This study has been limited to compounds of central sedative nature which, because of their pharmacological properties, would likely prove to be antagonistic to the convulsant and lethal effects of tetramine.

MATERIALS AND METHODS

Saturated aqueous solutions of tetramine preserved in wide-mouth colored glass bottles, from which appropriate quantities were removed by careful pipetting, have, in our hands, proved to be very appropriate for qualitative studies. Screening studies were first made on white rats which were given various sedative and convulsant medications subcutaneously, and later given 2 ml. (convulsant dose) or more of the saturated aqueous solution of tetramine intraperitoneally. These qualitative tests revealed that central depressant compounds administered in single doses would prevent or delay the tetramine-induced seizures, and certain ones would prevent death in severely intoxicated animals. The three most promising compounds revealed by these studies, out of a total of seven tested, were sodium bromide, sodium phenobarbital, and sodium barbital. Other compounds tested but not found to be sufficiently protective to warrant further study were chloral hydrate, urethane, sodium pentobarbital, and trimethadione.

Male Swiss white mice, weighing from 18 to 30 Gm., obtained from the National Laboratory Animal Co., Creve Coeur, Mo., were employed for a more extensive quantitative study of the effectiveness of sodium bromide, sodium phenobarbital, and sodium barbital in counteracting the intoxicating effects of tetramine. Mice were kept in stock at least seven days before use and were maintained on laboratory chow and water *ad libitum*.

Determination of Median Lethal Doses.—Varying dosages in aqueous solution of each antagonist drug, calculated as mg./Gm. weight of mouse were administered subcutaneously over the dorsum of the neck to at least six series of eight mice each. Solutions were prepared of such strength that volumes of fluid injected ranged from 0.20 to 0.60 ml. The percentage of deaths in each series was recorded and compared to a control series which received only a comparable volume of distilled water. The dose-effect curve for each antagonist compound was plotted and the LD_{50} and 95% confidence limits were obtained by statistical procedures outlined by Litchfield and Wilcoxon (8). The dose-effect curve for tetramine was also plotted by the same method. For this procedure we employed a stock solution of the drug in acetone, containing 1 mg./ml., which, before use, was diluted 1:20 with distilled water to make a final solution containing 25 mcg./ml. The LD_{50} for tetramine in mice was found to be 0.21 (0.19 to 0.23) mcg./Gm., or 0.21 mg./Kg. wt. A dose of 0.26 mcg./Gm. of tetramine killed all control series of unprotected mice, usually in two hours, and practically always within twenty-four hours after administration. This quantity was, therefore, selected as our "lethal dose" of tetramine.

The lethal dose of tetramine usually produced severe convulsive seizures in mice within five to twenty minutes after injection, and a good percentage of mice were dead within thirty minutes. This made it necessary to have all treated animals under sedation before tetramine was given. The electroshock seizure apparatus model 2-C[1] was used to determine a suitable time to allow for sedation in mice receiving sodium barbital and sodium phenobarbital. For sodium phenobarbital it was found that a dose of 15% of the LD_{50} was 100% effective in abolishing the extensor component of the supramaximal electroshock seizure (9, 10), in thirty minutes after intraperitoneal or subcutaneous injection; for sodium barbital, 30% of the LD_{50} produced this effect. Sodium bromide does not abolish the extensor component of the supramaximal electroshock seizure except in near lethal doses (10, 11). It was, however, found that 33% of the LD_{50} of this compound of sodium bromide was 100% effective in preventing convulsions in mice, when given subcutaneously twenty minutes before a dose of 12 ml./Kg. wt. of one-fourth saturated aqueous solution of tetramine given intraperitoneally. This dose of tetramine was 100% convulsive with two fatalities in a series of five controls. Since a period of twenty minutes was sufficiently long for absorption of anticonvulsant quantities of sodium bromide, it was concluded that a thirty-minute interval would be quite suitable for sodium bromide, as it was for sodium barbital and sodium phenobarbital. Thirty minutes was, therefore, chosen as the time interval between the subcutaneous injection of each antagonist and the intraperitoneal injection of tetramine solution.

Protective Dosage Curves.—Studies were next made on the effectiveness of each antagonist in reducing the fatalities resulting from intraperitoneal injection of 0.26 mcg./Gm. wt. lethal dose of

[1] Hans Technical Laboratories, Palo Alto, Calif.

tetramine. Various appropriately graduated doses of each antagonist were injected subcutaneously into series of eight mice for each dose, and thirty minutes later the lethal dose of tetramine solution was given intraperitoneally. Intoxicated animals were observed for as long as five days and the per cent of surviving (protected) animals in each series was tabulated. At least four doses between 0 and 100% protection were plotted (six doses in all) and by use of the Litchfield and Wilcoxon method (8) it was possible to plot protective dosage curves; for each antagonist, a median protective dose (PD_{50}) and 95% confidence limits were computed.

RESULTS

Results are summarized in Tables I and II. Table I lists the LD_{50} of tetramine and the three antagonists on white mice. Table II indicates that each compound affords protection against a lethal dose of tetramine when administered in a small percentage of its median lethal dose.

TABLE I.—TOXICITY OF SODIUM PHENOBARBITAL, SODIUM BARBITAL, SODIUM BROMIDE, AND TETRAMINE

Compound	Route	White Male Mice LD_{50} with 95% Confidence Limits, mg./Gm.
Sodium phenobarbital	s.c.	0.224 (0.204–0.245)
Sodium barbital	s.c.	0.785 (0.735–0.837)
Sodium bromide	s.c.	5.02 (4.76–5.29)
Tetramine, one-fourth saturated aqueous solution	i.p.	0.21 (0.19–0.23)

TABLE II.—PROTECTIVE EFFECTS OF SODIUM PHENOBARBITAL, SODIUM BARBITAL, AND SODIUM BROMIDE ON WHITE MICE GIVEN A LETHAL INTRAPERITONEAL DOSE OF TETRAMINE[a]

Antagonist[b]	Route	White Male Mice PD_{50} and 95% Confidence Limits, Dose, mg./Gm. Wt.	Per Cent of LD_{50}
Sodium phenobarbital	s.c.	0.015 (0.012–0.018)	6.7
Sodium barbital	s.c.	0.03 (0.024–0.038)	3.8
Sodium bromide	s.c.	0.25 (0.18–0.34)	5.00

[a] 0.26 mcg./Kg. wt. [b] Given subcutaneously.

DISCUSSION

The tetramine antagonists, even in relatively small doses, delayed time of onset and lessened the severity of convulsive seizures, or prevented death when appropriate doses were administered. The most essential activity for an effective pharmacological antagonist to tetramine would appear to be provided by compounds with prolonged central sedative and anticonvulsant action, since tetramine has been demonstrated to be very slowly metabolized. The more prolonged activity of the three compounds, sodium bromide, sodium phenobarbital, and sodium barbital, as compared to the shorter-acting compounds tested, namely, sodium pentobarbital, urethane, trimethadione, and chloral hydrate, might explain why they proved to be the more effective antagonists, when administered in single doses against tetramine. It is rather to be expected that when a single dose of a depressant is used to counteract a long-acting convulsant, a long-acting sedative and anticonvulsant should give the greatest protection because it allows the body a longer time to metabolize or excrete the intoxicating agent. In these studies no attempt was made to determine any increased effectiveness which might result from multiple injections of the antagonists. It is quite possible that repetitions in proper dosage and at properly spaced intervals might increase their effectiveness.

In case of accidental intoxication with tetramine in man, the administration of either of the three antagonists reported in this investigation would appear to be indicated. Further study might, however, reveal that sodium barbital and sodium bromide would be more effective than sodium phenobarbital in antagonizing the lethal effects of tetramine.

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Dilatometric Determination of Relatively Pure Systems

By A. P. SIMONELLI† and T. HIGUCHI

Theoretical and experimental analysis of volume-temperature curves of thymol containing naphthalene as the impurity shows that dilatometry can be applied to purity determinations of organic substances. Although the technique is essentially founded on the same phenomena as the freezing curve method and is necessarily somewhat slower, it is shown to be basically more elegant and more sensitive.

ALTHOUGH the theoretical feasibility of a dilatometric determination of absolute purity of many substances has been recognized for some time (1), there appears to be only limited earlier attempts to apply the method in practice. The present communication is concerned with the results of theoretical and experimental investigations of the dilatometric procedure for this purpose.

THEORY

A dilatometric curve consists of a plot of the volume of a substance *vs.* temperature, including a temperature range which spans from a temperature lower than the melting point to a temperature above the melting point. In general, the volume of a substance will increase with an increase in the temperature up to the melting point; here, the sample normally exhibits a sharp increase in volume due to melting. Dilatometric melting curves of pure or nearly pure substances are characterized by three parts. Figure 1 illustrates an idealized curve of a fairly pure sample, the line from A to B representing the expansion of the solid phase, the line from B to D the melting dilation, the line from D to E the expansion of the liquid phase, and B to C the melting point range of the sample.

It is obvious that if the melting temperature of the pure material is known the absolute purities of less pure samples can be easily determined. It is usually the situation, however, that only the sample is at hand and its purity is desired. In the following development based in part on the work of Glasgow, *et al.* (2), on freezing diagrams the melting point of the pure material is not needed.

Thermodynamics show that the melting point of a pure substance is lowered by an impurity, and the melting point depression is proportional to its concentration. White (3) proposed from a solid-liquidus phase diagram of a eutectic mixture that the concentration of the impurity will be highly concentrated in the beginning melt, and will be diluted as the compound continues to melt. This accounts for the fact that the melting point range of a compound increases with the addition of impurity. If the above is true, it predicts a hyperbola for the melting portion of the dilatometric curve, and this indeed, seems to be the case in the majority of the curves reported in the literature.

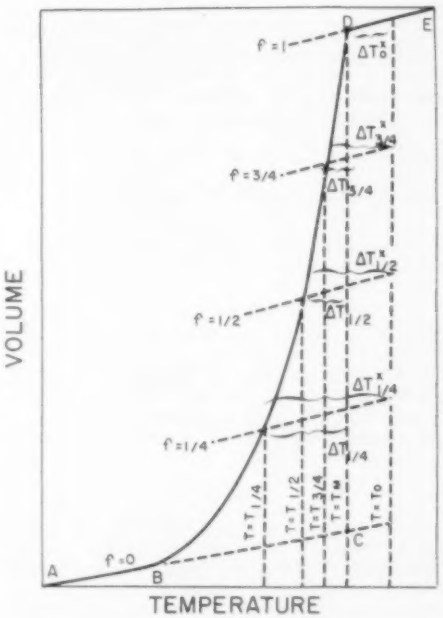

Fig. 1.—Theoretical analysis of a dilatometric melting curve.

The thermodynamic polynomial (2) which relates the mole fraction of impurity and the melting point depression is

$$N_2\left[1 + \frac{N_2}{2} + \frac{N_2^2}{3} + \ldots\right] = A_0\,\Delta T\,[1 + B_0\Delta T + C_0\Delta T^2 + \ldots]$$

where N_2 is the mole fraction of the impurity in the melt; A_0 is equal to $[\Delta H_0]/[RT_0^2]$; ΔH_0 is the heat of fusion of the major component in the pure state at the freezing point, T_0; R is the gas constant; ΔT is the freezing point depression which is equal to the difference in melting points of the pure substance and the sample; B_0 is equal to $[1/T_0 - \Delta C_p/2\Delta H_0]$; ΔC_p is the heat capacity of the liquid state less the heat capacity of the solid state for the major component; and C_0 is equal to $[1/T_0^2 - 2\Delta C_p/(3\Delta H_0 T_0)]$. The terms $(N_2^2/3)$ and higher for small values

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of N_2 and the terms $B_0\Delta T$ and higher for small values of ΔT can be omitted without introducing a large error. It follows that at the freezing point of the mixture

$$N_2^\circ\left[1+\frac{N_2^\circ}{2}\right]=A_0\,[T_0-T_m] \quad \text{(Eq. 1)}$$

where N_2° is the mole fraction of the impurity in the mixture, and T_m is the freezing point of the mixture. Similarly at any other temperature on the melting curve

$$N_2\left[1+\frac{N_2}{2}\right]=A_0[T_0-T_f] \quad \text{(Eq. 2)}$$

where N_2 is the mole fraction of the impurity in the melt, and T_f is the equilibrium temperature of the mixture at the fraction melted, f. If N_2° is small and considerations are restricted to the upper portion of the curve, it also can be shown that:

$$N_2^\circ=fN_2 \quad \text{(Eq. 3)}$$

Solution of Eqs. 1, 2, and 3 for N_2° yields

$$N_2^\circ=\frac{f}{1-f}\left\{\sqrt{1+\frac{2A_0[1+f]}{[1-f]}\,[\Delta T_f]}-1\right\} \quad \text{(Eq. 4)}$$

which is the desired expression in its final form. ΔT_f is equal to $[T_m - T_f]$.

A simpler expression can be derived if N_2° is sufficiently small so that the linear expressions, $N_2^\circ = A_0[T_0 - T_m]$ and $N_2 = A_0[T_0 - T_f]$, can be used instead of the quadratic expressions. For this case solution for N_2° yields

$$N_2^\circ=A_0\left[\frac{f}{1-f}\right]\Delta T_f \quad \text{(Eq. 5)}$$

Equations 4 and 5 can be applied to a dilatometric melting curve in the following manner. It is evident from Fig. 1 that the freezing point of the mixture can be obtained from the intersection of the melting portion of the curve and the liquid expansion portion of the curve.

The value of f can also be obtained from the plot. Since the sample is completely melted at the freezing point, f must be equal to unity at this point. If the solid expansion portion of the curve is extrapolated to the melting temperature, it is obvious that f equals zero along this line. This means that the difference in volume between the extrapolated solid portion of the curve and the liquid portion of the curve must correspond to the dilation due to melting; in addition, the difference in volume between any point on the curve and the extrapolated solid portion of the curve is a measure of the degree of melting which has taken place. The ratio of the two differences will be the value of f at the chosen temperature, T_f. This is illustrated by Fig. 1. At this point it is important that the difference between ΔT_f and ΔT_f° be clearly pointed out. ΔT_f° refers to the freezing point depression taken from the freezing point of the pure major component, i. e. $[T_0 - T_f]$, at the point f. ΔT_f (without the $^\circ$ superscript) refers to the freezing point depression taken from the freezing point of the mixture, i. e. $[T_m - T_f]$, at the point f. Figure 1 illustrates ΔT_f, ΔT_f°, and T_f for $f = 0$, $^1/_4$, $^1/_2$, $^3/_4$, and 1.

It is evident that certain assumptions were necessary to validate these relationships. They include (*a*) N_2 is sufficiently small so that the elimination of higher terms involving N_2 is valid, (*b*) ΔT_f is sufficiently small so that $[(1/T_0) - (\Delta C_p/2\Delta H_0)]\Delta T_f$ and higher terms can be eliminated, (*c*) the application of all relationships be restricted to the upper portion of the melting curve, (*d*) the system be at equilibrium (stirring was introduced for this purpose), (*e*) there are no solid occlusions present (this problem is eliminated in this study as all ingredients are weighed and added to the dilatometer separately), and (*f*) the system exhibits ideal behavior. An ideal system is one in which: (*a*) there are no solid solutions present, (*b*) the impurity forms an ideal solution with the melt, (*c*) the heat content of the melt is independent of the impurity and a function of the temperature only, (*d*) the dilation coefficient (dilation due to melting only) remains constant throughout the melting range, (*e*) the heat capacity of the liquid and solid phases of the major component is constant over the melting range, and (*f*) the impurity is miscible in the melt. If a system does not exhibit idealistic behavior, errors will be introduced in the estimation of its purity. In general, however, the equations can be applied to nonideal as well as ideal systems if the value of A_0 is determined from a dilatometric curve and the same value of f is used to determine ΔT_f in both the known and unknown curves.

It must be noted that the impurity can consist of more than one species because the relationships are defined in quantities characteristic of the solvent only, and are not affected by the nature of the solute.

EXPERIMENTAL

The apparatus essentially consisted of a water bath, dilatometer, thermometers, and optical micrometers.

Water Bath.—Since the temperature is a variable appearing in all of the equations, it must be rigidly controlled. For this purpose a water bath was designed so that the temperature was controlled to ±0.001° and could be easily varied from 0 to 100°. It utilized a continuous type of control with a thermistor as a sensing unit, an infrared lamp as a heating element, and high speed stirring to insure a uniform temperature throughout the bath. To minimize the rate of heat loss a sealed cover was used to inhibit evaporation, the room temperature was controlled so that the variance was less than 1°, and an insulating jacket was wrapped about the bath jar.

Dilatometer.—A volumetric 2-mm. bore dilatometer was used (4). The height of the mercury in the constant bore tubing of the dilatometer was obtained by using an optical micrometer. Changes in height of the mercury in the constant bore tubing are directly proportional to changes in volume of the system as the volume of any segment of the constant bore tubing is equal to the cylinder described by $[\pi r^2\Delta\text{height}]$ where r is the radius of the bore and Δheight is the change in mercury height. The sensitivity of the volume determined in this way will be equal to the volume of the cylinder formed in the

bore of the dilatometer with the sensitivity of the micrometer as the height; i. e., $\pi r^2 \Delta$height. Since the height of the mercury could be estimated to a micron, the uncertainty in the determination of a change in volume is approximately 0.000003 ml.

Stirring of Sample.—It was felt that stirring is necessary in order to achieve the required equilibrium. For this purpose a seamless Teflon-covered stirring bar magnet was machined to fit the small opening in the dilatometric chamber. During a run the stirring bar magnet in the dilatometer chamber was moved in a linear vertical motion by means of a 5-lb. horseshoe magnet which was placed outside of the dilatometer chamber and raised and lowered by means of a reduction motor. To prevent a high incidence of breakage during stirring a metal bracket was designed so as to eliminate pressure upon the more delicate parts of the dilatometer.

Procedure.—The sample and the stirring bar were placed in the dilatometer and degassed under vacuum overnight. Mercury was then introduced into the dilatometer which was then placed into the water bath at a temperature below the melting point range of the sample. The height of the mercury in the constant bore tubing of the dilatometer was obtained as a function of the temperature by increasing the water bath temperature and observing the height of the mercury until it remained constant with time. At this point the sample was assumed to be at equilibrium. After recording the height of the mercury and the water bath temperature, the temperature of the water bath was again increased. This procedure was repeated at small increments of temperature and extended over a sufficient temperature range so as to define accurately the dilatometric melting curve of the sample. When the temperature of the water bath was increased to the point that the sample would start to melt, stirring was initiated to decrease the possibility of obtaining a false equilibrium.

Obtaining the Expression Due to Melting.—Since it was desirable that only the expansion due to melting be considered, it was necessary to separate the expansion due to the dilatometer, stirrer, and the mercury from the expansion due to the sample; furthermore, it would also be desirable to eliminate the expansion of the solid phase. In order to eliminate time-consuming calibrations and calculations which would not contribute anything important to this discussion, a graphical approach was used. If the solid expansion portion of the curve illustrated in Fig. 1 is extrapolated from AB to C, then, the volume represented by the area beneath this extrapolated curve is due to the expansion of the solid phase, the stirrer, the dilatometer, and the mercury. The volume represented by the difference in area of the two curves will be, for all intents and purposes, due to the dilation because of melting. For convenience, the dilatometer readings expressed as heights of the mercury column were not converted to volumes but to fractional degrees of melting.

RESULTS AND DISCUSSION

Dilatometric melting curves obtained for thymol containing 0.0004, 0.0102, 0.0224, 0.0370, 0.0627, and 0.1155 mole fractions of impurities are shown in Fig. 2. The mole fraction of impurity in the supposedly pure thymol used was estimated to be 0.0004 or 99.96% pure (see Table I), and sufficient naphthalene was added to increase the impurity to these levels. Thymol of the above purity was obtained by distilling thymol U.S.P. and then recrystallizing it from three different solvents. The purification was followed by applying Eq. 4 to a dilatometric melting curve of the sample after each purification. The naphthalene used in this study was purified by sublimation.

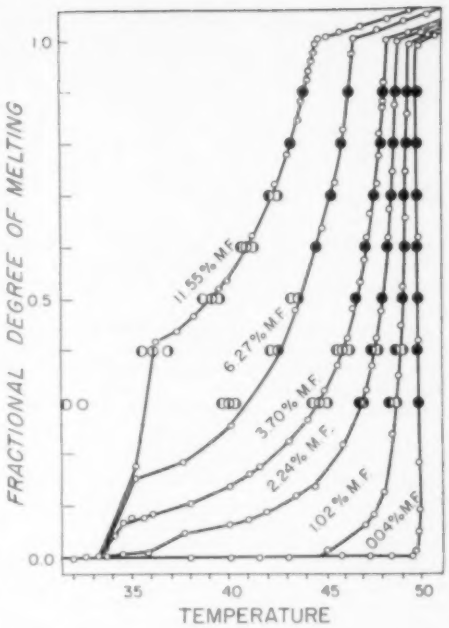

Fig. 2.—Plots of the theoretical and experimental data in which the heights of mercury have been converted to fractional degrees of melting. O, Experimental points for all curves; ⊖, theoretical points calculated from Eq. 4; ◐, theoretical points calculated from Eq. 5; ◑, theoretical points calculated from Eq. 7.

Thymol was selected as the major component because (*a*) it is readily available, (*b*) its melting point presents no experimental difficulties, (*c*) it is readily purified, (*d*) dilation coefficient is sufficiently large, (*e*) it is sufficiently stable in the range of temperature of the experiment, and (*f*) it is inert toward mercury, glass, and Teflon.

Estimation of Purity.—Equation 4 or 5 can be used to determine the mole fraction of impurity as the cryoscopic constant, A_0, can be obtained from the literature, and ΔT_f at any particular fraction melted can be obtained from a dilatometric curve of the compound.

If the heat of fusion for thymol (5) is taken as 17.3 Kilojoules per mole at 48.5°, the value of A_0 is 0.02011. Based on both Eqs. 4 and 5, the per cent mole fraction of impurity in the total sample calcu-

TABLE I.—VALUES OF % N_2^* CALCULATED FROM EQS. 4 AND 5 AT DIFFERENT VALUES OF f

	At f = 0.75			At f = 0.80				At f = 0.85		
		% N_2^*	% N_2^*		% N_2^* of Eq. 4		% N_2^* of Eq. 5		% N_2^*	% N_2^*
Sample	$\Delta T_{0.75}$	Eq. 4	Eq. 5	$\Delta T_{0.80}$	A_0 Lit.	A_0 Expt.	A_0 Lit.	A_0 Expt.	$\Delta T_{0.85}$	Eq. 4	Eq. 5
0.04	0.006	0.036	0.036	0.005	0.040	...	0.040	...	0.003	0.034	0.034
1.02	0.16	0.95	0.97	0.13	1.03	1.17	1.05	1.14	0.10	1.13	1.14
2.24	0.37	2.18	2.23	0.30	2.35	2.55	2.41	2.62	0.23	2.55	2.62
3.70	0.57	3.31	3.44	0.42	3.26	3.65	3.38	3.67	0.295	3.25	3.36
6.27	0.95	5.39	5.73	0.71	5.38	6.05	5.71	6.21	0.50	5.38	6.00
11.55	1.81	9.66	10.92	1.35	9.72	10.87	10.86	11.80	0.95	9.79	10.83

lated from ΔT_f data at several values of f are shown in Table I. The results suggest that purity can be determined within a few parts per thousand by this procedure.

In the event A_0 is not known, a standard mixture can be used. According to Eq. 5 the ratio of impurity of two mixtures at the same f and containing the same major component is

$$\frac{[N_2^*]_x}{[N_2^*]_y} = \frac{[\Delta T_f]_x}{[\Delta T_f]_y} \qquad \text{(Eq. 6)}$$

where the subscript x corresponds to the unknown mixture and y the standard. In order to determine the unknown mole fraction of impurity, ΔT_f must be secured from a dilatometric curve of both the known and unknown at the same fraction melted. Since $[N_2^*]_y$ is known, $[N_2^*]_x$ can be calculated. Equation 6 can be used, however, only if the impurity of the unknown is not large. Using Eq. 6 the mole fraction of impurity was calculated for all the samples using the sample containing 0.0102 N_2^* as the standard. This also was repeated using the other samples as standards. The results are listed in Table II and they reveal that the purity can be determined within parts per thousand if a standard is available.

TABLE II.—VALUES OF % N_2^* CALCULATED FROM EQ. 6 AT f = 0.8

	Samples as Standards				
Sample	1.02	2.24	3.70	6.27	11.55
1.02	1.02	0.97	1.15	1.15	1.15
2.24	2.35	2.24	2.64	2.65	2.59
3.70	3.30	3.14	3.70	3.71	3.62
6.27	5.57	5.30	6.25	6.27	6.12
11.55	10.51	10.01	11.80	11.83	11.55

The value of A_0, however, can be calculated from experimental data using Eq. 4 or 5 as the values of ΔT_f, and f can be obtained from a dilatometric melting curve of a standard mixture of known mole fraction of impurity, N_2^*. This value of A_0 can then be used to determine the purity of samples which contain relatively greater amounts of impurity. The sample containing 0.0370 mole fraction of impurity was used to determine the value of A_0 experimentally. Ignoring the original impurity, as it would not be known if A_0 was not available, N_2^* was taken to be 0.0367 which was the amount of naphthalene added. The value of A_0 was found to equal 0.02275 using Eq. 4 and 0.02185 using Eq. 5. Using the respective values of A_0, N_2^* was determined at f = 0.8, using both equations and the results tabulated in Table I where they can be compared with the values of N_2^* obtained using the literature values of A_0. The results seem to indicate that the value of ΔH_0 need not be available to apply this theory for purity determinations if the major component can be sufficiently purified.

Prediction of Dilatometric Curve.—If N_2^*, A_0, and the freezing point of the sample is known, Eq. 4 or 5 permits the prediction of the melting portion of a dilatometric curve since ΔT_f can be calculated for any f.

If the freezing points of both the pure major component and mixture is known, the melting portion of a dilatometric curve of the mixture can be predicted even if N_2^* and A_0 are not known since

$$\Delta T_f^* = \frac{\Delta T_0^*}{f} \qquad \text{(Eq. 7)}$$

where ΔT_0^* is the freezing point depression of the mixture at T_m, i. e., $T_0 - T_m$, and ΔT_f^* is the freezing point depression of the mixture at T_f, i. e., $T_0 - T_f$. Equation 7 was derived by starting with the linear relationships to obtain the ratio N_2^*/N_2, substituting, f for N_2^*/N_2, and rearranging.

An inspection of Fig. 2 reveals that the theoretical points predicted by Eqs. 4, 5, and 7 are in excellent agreement with the experimental curves. This is particularly true at the upper portions of the curve. Figure 2 also reveals that the 2.24, 3.7, 6.27, and 11.55% mole fraction of impurity curves showed a break in the curve at about 33°. It is believed that these breaks are due to the presence of increased amounts of impurities as no breaks appeared in the samples of higher purity; in addition, the height of the break was proportional to the concentration of the impurity. This behavior indicates that naphthalene is not miscible with thymol in all proportions. In the event of limited solubility, theory would predict that the impurity would form a eutectic with the major component as demonstrated in this system.

Freezing Point of Pure Major Component.—The freezing point of the pure major component can also be obtained from the dilatometric melting curve of a sample. An inspection of Fig. 1 shows that $\Delta T_f^* = \Delta T_f + \Delta T_0^*$. If this is substituted into Eq. 7 and the result rearranged, the following expression is obtained

$$T_0 = T_m + \left[\frac{f}{1-f}\right]\Delta T_f \qquad \text{(Eq. 8)}$$

Equation 8 shows that the freezing point of the pure major component can be calculated as the values of all unknowns can be obtained from a dilatometric curve of the mixture. Using Eq. 8, the freezing point of pure thymol was calculated from all curves at different values of f, and the values were listed in Table III.

Ideality of the System.—The ideality of the system studied can also be determined. Since Eq. 5 is a linear relationship, a plot of T_f *vs.* $[1 - f]/f$

TABLE III.—FREEZING POINT OF PURE THYMOL CALCULATED FROM EQ. 8

Sample	Values of T_0 Calculated at Values of f						
	0.50	0.60	0.70	0.75	0.80	0.85	0.90
1.02	49.79	49.82	49.85	49.89	49.95	49.93	49.95
2.24	49.87	49.91	50.01	50.02	50.15	50.25	50.26
3.70	49.79	49.86	49.88	49.86	49.83	49.85	49.77
6.27	49.16	49.38	49.23	49.30	49.32	49.31	49.45
11.55	50.10	49.88	49.80	49.98	49.91	49.93	50.04

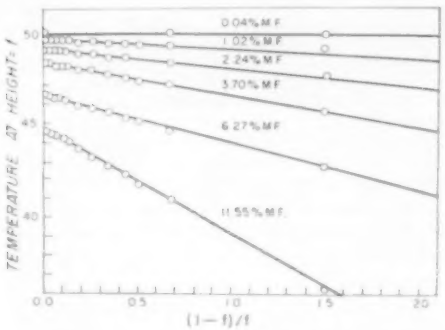

Fig. 3.—T_f *vs.* $[1 - f]/f$ curves for all samples. The line represents the theoretical curve from Eq. 5 and the circles are the experimental points.

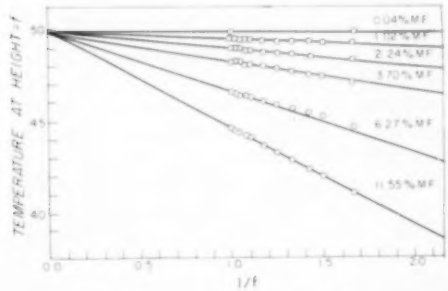

Fig. 4.—T_f *vs.* $1/f$ curves for all samples. The line represents the theoretical curve from Eq. 5 and the circles are the experimental points.

will be a straight line if N_2^* is sufficiently small and the system exhibits ideal behavior. Curvature of this plot, then, would provide a measure of the nonideality of the system. Figure 3 shows the plots of T_f *vs.* $[1 - f]/f$ for all the samples used. The straight lines are the theoretical curves using the freezing point of each sample as the intercept and drawing a line with a slope equal to the freezing point depression, $T_0 - T_m$. The experimental points show excellent agreement with the theoretical curve.

Equation 7 which is a hyperbola can be converted to the linear relationship if $T_0 - T_f$ is substituted for ΔT_f^*

$$T_f = T_0 - \Delta T_0^*/f \qquad \text{(Eq. 9)}$$

Equation 9 predicts that a plot of T_f *vs.* $1/f$ will yield a straight line. Curvature of this plot also would provide a measure of the nonideality of the system studied. Figure 4 shows the plots of T_f *vs.* $1/f$ for all samples used. The straight lines are the theoretical curves using the theoretical freezing point of pure thymol as the intercept and drawing a line with a slope equal to the freezing point depression of the sample.

The experimental points show excellent agreement with the theoretical curve, and this seems to indicate that a sample of naphthalene and thymol exhibits ideal behavior at low concentrations of naphthalene.

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Cyclized Substituted Thioureas I

Preparation of Some 3-Substituted 1,2,3,4-Tetrahydroquinazoline-2-thiones and Their Intermediates

By RONALD E. ORTH† and JAMES W. JONES

Some 3-substituted 1,2,3,4-tetrahydroquinazoline-2-thiones were prepared by first synthesizing the N-substituted-*o*-nitrobenzylamines, then the corresponding N-substituted-*o*-aminobenzylamines. The tetrahydroquinazoline-2-thiones were then obtained by refluxing the diamine with carbon disulfide. It is suggested that antithyroidal, antitubercular, and/or antifungal properties might be present in these condensed ring thiones.

THE THERAPEUTICALLY active antithyroid drugs marketed today contain the thiourea moiety. It is demonstrable that to obtain appreciable activity at least one of the nitrogen atoms of the moiety has an attached hydrogen atom. Recent work leads to the belief that toxicity is reduced when the thiourea group is incorporated into certain ring systems and one of the nitrogens is substituted. The purpose of this investigation was to prepare some active, nontoxic heterocycles which incorporate the active thiourea moiety.

No quinazoline pharmaceuticals are available at this time, however, some alkaloids which contain this nucleus are known. Peganine; the carboline alkaloids rutecarpine and evodiamine; febrifugine, which has a high antimalarial activity and toxicity; and dichroine, a febrifuge from the roots of *dichroa febrifuga* Lour, are a few. The latter has been used in China for centuries under the name of Ch'ang Shan (1). 2-Amino-5,6,7,8-tetrahydroquinazoline has given slight protection to mice against β-hemolytic streptococcus (2). The 2- and 4-chloroquinazolines and derivatives condensed with a wide variety of aromatic amino, hydroxyl, and sulfhydryl compounds containing chromophoric groups have been used in the dye industry (3). U. S. patent 2,400,649 (4) describes the preparation of N-[4-(4-quinazoline)-aminobenzoyl]-glutamic acid which is a relative of folic acid and is claimed to be useful as a vitamin and chemotherapeutic agent. The partial synthesis of the quinazoline analog of papaverine has been reported by Marr and Bogert (5). The majority of the work involving the quinazoline nucleus has been in the field of antimalarials. In 1919 Wilkendorf (6) synthesized some quinazoline analogs of 8-aminoquinoline (pamaquine) and in 1924 Bogert, *et al.* (7), made an analog of cinchophen, 2-phenyl-quinazoline-4-carboxylic acid. Rodionov, *et. al.* (8), synthesized a group of 3-(dialkylaminoalkyl)-4-quinazolones. Christensen, *et al.* (9), prepared 2,4-dimethyl-7-(1-hydroxy-3-amino-4-hydroxy- and 2,4-diamino-5,6,7,8-tetrahydroquinazolines). Price, *et al.* (10), made quinazoline analogs of 4-aminoquinoline (chloroquine). In 1946 Bunnett, *et al.* (11), made antimalarials having the general formula 2-(dialkylaminoalkylamino)-4-quinazolone. A number of N′-quinazolylsulfanilamides were synthesized by MacBeth and Rodda (12) but they were too insoluble to be active as antimalarial agents. The above references are evidence that, although the quinazolines are not common in human medication, they have been of interest for many years.

The 3-substituted 1,2,3,4-tetrahydroquinazoline-2-thiones were synthesized by first preparing α-bromo-*o*-nitrotoluene by the method of Kornblum (13). A number of N-substituted *o*-nitrobenzylamines were formed and characterized by their hydrochloride salts, as shown in Table I. The *o*-nitro groups were reduced to *o*-amino groups and the resulting compounds were characterized as dihydrochlorides, as shown in Table II. These diamines were refluxed with carbon disulfide causing ring closure and the formation of the corresponding 3-substituted tetrahydroquinazoline-2-thiones. See Table III.

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EXPERIMENTAL

N-Ethyl-*o*-nitrobenzylamine Hydrochloride. Twenty milliliters of 70% aqueous ethylamine (0.3 mole) and 0.03 mole of α-bromo-*o*-nitrotoluene were dissolved in 50 ml. of 95% ethanol and shaken in a sealed flask for one hour. The mixture then was set aside at room temperature for eight days, after which it was filtered. The clear filtrate was evaporated on a steam bath until a semisolid (oil) formed. This semisolid was treated with 200 ml. of dilute hydrochloric acid which produced a clear solution. The water was removed on the steam

TABLE I.—HYDROCHLORIDE SALTS OF N-ALKYL- AND N-ARYL-*o*-NITROBENZYLAMINE

R	C, %		H, %		N, %		Yield, %	M. P., or Decompn., Pt., °C.
	Calcd.	Found	Calcd.	Found	Calcd.	Found		
Methyl	47.6	48.1	5.45	6.00	13.9	14.1	22	185–187[b]
Ethyl	50.0	50.7	6.02	6.12	13.0	13.7	28	223–225[b]
n-Propyl	52.1	53.1	6.54	6.84	12.2	12.8	18	138[b]
Isopropyl	52.1	51.9	6.54	6.57	12.2	12.0	47	255[b]
Allyl	52.6	51.7	5.70	5.44	12.3	11.7	17	168–170[b]
Isobutyl	54.1	54.4	6.97	7.21	11.5	11.2	52	189[b]
t-Butyl	54.1	53.9	6.97	6.89	11.5	11.5	63	173–174[b]
Phenyl	59.0	59.5	4.91	4.93	10.6	10.9	73	182–184[b, c]
Benzyl[a]	69.5	69.5	5.30	5.47	11.6	11.1	87	225
Cyclohexyl	60.5	59.8	7.41	7.55	10.9	10.7	45	250–252[b]

[a] Free base. [b] Decomposed. [c] Literature 140–145°.

TABLE II.—DIHYDROCHLORIDE SALTS OF *o*-AMINO-N-ALKYL- AND N-ARYLBENZYLAMINE

R	C, %		H, %		N, %		Yield, %	M. P., or Decompn. Pt., °C.
	Calcd.	Found	Calcd.	Found	Calcd.	Found		
Hydrogen	42.9	42.7	6.15	6.31	14.4	...	97	167[b]
Methyl	45.8	44.8	6.70	6.51	13.4	...	96	217[b]
Ethyl	48.4	48.8	7.16	7.00	12.5	12.2	78	210[b]
n-Propyl	50.8	50.5	7.63	8.15	11.8	11.7	51	275[b]
Isopropyl	50.8	50.7	7.63	7.99	11.8	11.6	63	191[b]
Allyl	51.0	50.2	6.84	6.79	11.9	11.7	58	163[b]
Benzyl	59.0	59.3	6.32	6.66	9.8	...	87	210[b]
Phenyl[a]	78.7	77.9	7.06	7.42	14.2	...	90	86
Cyclohexyl	56.3	55.9	7.94	7.87	10.1	9.95	54	234[b]

[a] Free base. [b] Decomposed.

TABLE III.—3-ARYL- AND 3-ALKYL-1,2,3,4-TETRAHYDROQUINAZOLINE-2-THIONES

R	C, %		H, %		N, %		Yield, %	M. P., or Decompn., Pt., °C.
	Calcd.	Found	Calcd.	Found	Calcd.	Found		
Hydrogen	58.2	57.7	4.87	4.22	17.0	...	43	212[a]
Methyl	60.5	58.9	5.63	5.61	15.7	...	48	181[a]
Ethyl	62.5	62.1	6.25	5.95	14.6	14.7	31	185[a]
Isopropyl	64.0	63.3	6.80	6.71	13.6	13.3	24	110
Phenyl	70.0	69.2	5.00	5.11	11.7	...	87	212[a]
Cyclohexyl	68.2	68.4	7.32	7.21	11.4	11.5	39	147[a]
Benzyl	71.5	70.9	5.56	5.81	11.1	11.7	43	112

[a] Decomposed.

bath leaving an amber, crystalline mixture. Dilute sodium hydroxide, 300 ml., was added to liberate the free oily base. The oil was separated from the aqueous layer and picked up in ether. The ether solution was dried over anhydrous sodium sulfate and filtered. The filtrate was evaporated to dryness and the residue dissolved in 200 ml. of dilute hydrochloric acid. Upon evaporation to dryness, pale yellow crystals of the hydrochloride of N-ethyl-*o*-nitrobenzylamine were obtained. Washing with small quantities of ether gave small, white crystals which melted at 223–225°.

N-*n*-Propyl-*o*-nitrobenzylamine Hydrochloride.—Two-tenths mole of *n*-propylamine and 0.03 mole of *α*-bromo-*o*-nitrotoluene were dissolved in 70 ml. of 95% ethanol. The procedure used for preparing N-ethyl-*o*-nitrobenzylamine hydrochloride was followed here. White crystals, melting at 138° were obtained.

N-Isopropyl-*o*-nitrobenzylamine Hydrochloride.—The same procedure as used in preparing the ethyl analog was followed starting with 0.25 mole of isopropylamine and 0.03 mole of the *α*-bromo-*o*-nitrotoluene dissolved in 110 ml. of 95% ethanol. However, the impurities were best eliminated by washing first with absolute ether, then with absolute acetone, and finally with absolute alcohol. Recrystallization was not used because of the small quantities dealt with here. The substance had a white, cubical, crystalline appearance. It sublimed at 200° and melted at 255°.

N-Allyl-*o*-nitrobenzylamine Hydrochloride.—One-fourth mole of allylamine and 0.03 mole of *α*-bromo-*o*-nitrotoluene in 70 ml. of 95% ethanol were shaken for six hours. The procedure then followed that used for the ethyl derivative. A small yield of white crystals, which decomposed at 168–170° was obtained.

N-Isobutyl-*o*-nitrobenzylamine Hydrochloride.—One-fourth mole of isobutylamine, which boils at 68–69°, was refluxed with 0.03 mole of *α*-bromo-*o*-nitrotoluene for twelve hours in ethanol to complete the reaction. Cooling the reaction mixture resulted in a dark solution. The ethanol was distilled off leaving a very thick, viscous oily amine. The amine was picked up in absolute ether, dried over anhydrous sodium sulfate and filtered. The ethereal filtrate was evaporated to obtain the amine. The amine was then treated with 10% hydrochloric acid and the resulting solution was evaporated to dryness. The crystals melted at 189°.

N-*t*-Butyl-*o*-nitrobenzylamine Hydrochloride.—A solution consisting of 0.25 mole of tertiary-butylamine, 0.03 mole α-bromo-*o*-nitrotoluene, and 80 ml. of ethanol was refluxed for two hours. The procedure followed for the isolation of the isobutyl derivative was used. The large, white, rectangular crystals decomposed at 173–174°.

N-Benzyl-*o*-nitrobenzylamine.—One-fourth mole of benzylamine and 0.03 mole of α-bromo-*o*-nitrotoluene were dissolved in 50 ml. of 95% ethanol and refluxed twelve hours. Using the same procedure as that for preparing the isobutyl derivative, white platelets melting at 225° were obtained.

N-Cyclohexyl-*o*-nitrobenzylamine Hydrochloride.—One-fourth mole of α-bromo-*o*-nitrotoluene was shaken with 0.2 mole of cyclohexylamine until the exothermic reaction seemed to be complete. It was then placed on a steam bath for one-half hour to insure complete reaction. The mixture was then extracted with five 100-ml. portions of ether. The ether extracts were dried over sodium sulfate and filtered. The filtrate was distilled under vacuum to remove the ether and any remaining unreacted primary amine. The oil remaining was treated with 10% hydrochloric acid to form a salt. The salt was taken up in hot 95% ethanol and precipitated as light yellow crystals upon cooling. The crystals were reprecipitated from ethanol and washed with absolute ether. The crystals decomposed at 250–252°.

N-Phenyl-*o*-nitrobenzylamine Hydrochloride.—Three-hundredths of a mole of α-bromo-*o*-nitrotoluene and 0.2 mole of aniline were heated together on a steam bath with constant agitation for one-half hour. The reaction mixture solidified upon cooling. The solid was treated with 36% acetic acid which dissolved all but the heavy, dark brown oily secondary amine. The two liquids were separated and the oily amine was treated with 10% hydrochloric acid, after which, the solution was evaporated to dryness on a steam bath. The impure crystalline mass was recrystallized from boiling gracial acetic acid. The resulting crystals were then washed with absolute ether giving long, fine, white, needle-like crystals which decomposed at 182–184°. The salt was previously reported to decompose at 140–145° (14). Lellman (15) prepared the free amine by another method and found the melting point to be 44°.

N-Phenyl-*o*-aminobenzylamine.—This amine was prepared by dissolving 0.025 mole of N-phenyl-*o*-nitrobenzylamine hydrochloride in glacial acetic acid on a magnetic stirrer. Small portions of zinc dust were added keeping the temperature always below 30° (preferably below 25°), until 13.2 Gm. of zinc was completely dissolved. The solution turned from yellow to transparent when all of the zinc was dissolved. Water was added in small portions from time to time in order to keep zinc acetate from precipitating. After the reduction had been carried out, concentrated sodium hydroxide solution was added until the solution was nearly neutral. At this point, colorless crystals of the base precipitated out. They were recrystallized from hot ethanol. The resulting crystals melted at 81°.

***o*-Aminoalkyl- and Arylbenzylamine Dihydrochlorides.**—Five grams of the N-alkyl- or N-aryl-*o*-nitrobenzylamine hydrochloride was added in small portions to mixture of 10 Gm. of tin and 25 Gm. concentrated hydrochloric acid. The mixture was heated for one hour on a water bath to insure complete reduction. It was then diluted with 1 L. of water and filtered. The filtrate was made alkaline with 40% sodium hydroxide solution which produced a precipitate of tin hydroxide. The precipitate was filtered out and the filtrate extracted with several small portions of ether. The ether extract was dried over anhydrous sodium sulfate and filtered. The ether was evaporated, leaving the impure amine. The amine was treated with 10% hydrochloric acid solution and evaporated to dryness. The hydrochloride salt was then recrystallized from alcohol-ether and washed with absolute acetone.

3-Aryl- and 3-Alkyl-1,2,3,4-tetrahydroquinazoline-2-thiones.—Two grams of the free base *o*-amino-N-alkyl- or *o*-amino-N-arylbenzylamine, liberated from the hydrochloride by neutralization with 20% sodium hydroxide solution, was dissolved along with 20 Gm. of carbon disulfide in 50 ml. of absolute ethanol and refluxed for several hours on a water bath. The excess carbon disulfide and ethanol were then removed by distillation and the impure quinazolinethione was picked up in boiling absolute ethanol. Upon cooling, pure 3-aryl- and 3-alkyl-1,2,3,4-tetrahydroquinazoline-2-thiones precipitated.

SUMMARY

1. The following hydrochloride salts of N-aryl- and N-alkyl-*o*-nitrobenzylamine were prepared: methyl,[1] ethyl, *n*-propyl, isopropyl, allyl, isobutyl, *t*-butyl, phenyl,[1] and cyclohexyl. The free base was prepared for benzyl derivative.

2. The following dihydrochloride salts of *o*-amino-N-alkyl and *o*-amino-N-arylbenzylamine were prepared: methyl,[1] hydrogen,[1] ethyl, *n*-propyl, isopropyl, allyl, benzyl,[1] and cyclohexyl. The free base *o*-amino-N-phenylbenzylamine was prepared.

3. The following 3-aryl- and 3-alkyl-1,2,3,4-tetrahydroquinazoline-2-thiones were prepared: hydrogen,[1] methyl,[1] ethyl, isopropyl, phenyl,[1] cyclohexyl, and benzyl.

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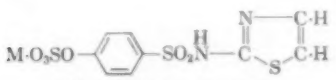
Mannich Bases of Phenolsulfonamides: Derivatives of N-(2-Thiazolyl)-1-phenol-4-sulfonamide

By GEORGE M. SIEGER, WILLIAM C. BARRINGER, FRANCIS M. CALLAHAN, NORBERT GRUENFELD, and JOSEPH F. WEIDENHEIMER

A series of Mannich base derivatives of N-(2-thiazolyl)-1-phenol-4-sulfonamide has been synthesized and characterized largely in the form of hydrochloride salts and acetyl derivatives. The results on a variety of biological screening tests carried out on these compounds have been reported.

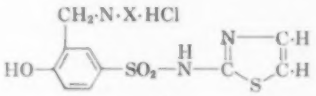
SOME TIME ago an interest was expressed by these laboratories in a series of phenolsulfonamides, namely, N-(2-thiazolyl)-1-phenol-4-sulfonamides[1] and analogs as a potential chemotherapeutic agent for certain neurotropic virus diseases (1–3, 7). More complete and exhaustive testing of these compounds gave indication that these compounds were not as effective against certain viruses as initially thought (8). However, before these biological results were reported, it was decided that water-soluble derivatives of the phenolsulfonamides should be prepared as a possible means of improving their pharmaceutical utility. One means of effecting water solubility was to prepare phenolic hemi-sulfuric esters of the phenolsulfonamides that could be isolated as metal and/or amine salts; this work was carried out by Hultquist and Webb (2). These compounds can be represented by general formula A.



M, metal or amine cation

A

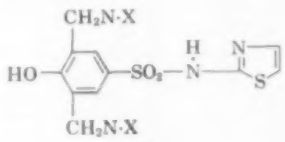
In these laboratories another approach to the problem was initiated at the time and some work has been pursued intermittently in this area over a period of years. It involved the synthesis of Mannich base derivatives which could be prepared and isolated as water-soluble acid salts, where ·N·X in the studies was represented by substituted secondary heterocyclic and dialkyl



B

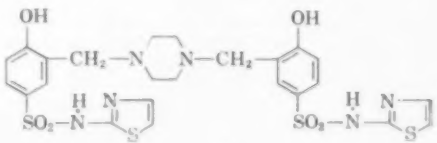
amines, e. g., morpholine, piperidine, pyrrolidine, piperazines, diethylamine, and dimethylamine.

The phenolsulfonamides, having both *ortho* positions to the phenolic hydroxyl group free and the *meta* directing sulfonamido group in the position *para* to the hydroxyl, have lent themselves readily to Mannich condensations. Other general types of compounds that theoretically could be derived from the Mannich condensations on N-(2-thiazolyl)-1-phenol-4-sulfonamide are: a di-Mannich base substituted into both positions *ortho* to the phenolic hydroxyl, i. e.,



C

and a Mannich base in which two phenolsulfonamide molecules are joined to both amine nitrogens of a di-functional amine, such as piperazine, by means of a methylene bridge, e. g.,



D

In order to identify and characterize these compounds, the Mannich bases were isolated as free bases, hydrochloride salts, and/or acetyl derivatives. In Table I are listed the compounds prepared, their respective identifying numbers, their names, melting points, yield, and micro-

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[1] N-(2-Thiazolyl)-1-phenol-4-sulfonamide is also known and tested under the names phenosulfazole and Darvisul.

TABLE I.—CHARACTERIZATION OF COMPOUNDS PREPARED

No.	Compound	Empirical Formula	Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Chlorine Calcd.	Chlorine Found	Nitrogen Calcd.	Nitrogen Found	Sulfur Calcd.	Sulfur Found	Approx. Yield, %	M. P. (with Decompn.) °C.	Crystallization Solvents
I	2-(4-Morpholinomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide	$C_{14}H_{17}N_3O_4S_2$	47.31	47.17	4.82	5.01	...	...	11.82	11.41	18.04	18.0	71.7	194–197	DMF[b]-H_2O
Ia	Hydrochloride	$C_{14}H_{18}ClN_3O_4S_2$	42.90	42.86	4.63	4.96	9.05	9.23	10.72	10.82	16.36	16.58	62.0	195–198	H_2O
Ib	Acetyl derivative	$C_{16}H_{19}N_3O_5S_2$	48.35	48.82	4.82	5.19	...	...	10.57	10.37	16.13	15.95	25.0	165–167	Acetone-H_2O
II	2-(Piperidinomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide	$C_{15}H_{19}N_3O_3S_2$	50.97	50.40	5.42	5.73	...	...	11.89	11.83	...	...	70.6	202–203	DMS[c]-EtOH
IIa	Hydrochloride	$C_{15}H_{20}ClN_3O_3S_2$	46.20	46.23	5.17	5.38	9.09	8.83	10.78	10.59	16.45	16.23	50.0	185–189	MeOH-EtOH-Ether
IIb	2,6-Bis compound dihydrochloride	$C_{21}H_{32}Cl_2N_4O_3S_2$	48.17	48.33	...	...	13.54	13.68	10.70	10.72	...	...	8.0	184–191	DMF-50-50 Iso-PrOH-Pet. Ether
III	2-(Pyrrolidylmethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide	$C_{14}H_{17}N_3O_3S_2$	49.54	49.15	5.05	5.09	...	...	...	...	18.89	18.50	73.7	193–196	DMS-EtOH
IIIa	Hydrochloride	$C_{14}H_{18}ClN_3O_3S_2$	44.73	45.00	4.83	4.88	9.43	9.22	11.18	11.33	17.06	17.7	40.0	204–206	EtOH-Ether
IV	N,N′-Di-[N-(2-thiazolyl)-5-sulfonamido-2-hydroxybenzyl] piperazine	$C_{24}H_{26}N_6O_6S_4$	46.29	45.84	4.21	4.48	...	...	13.50	13.15	20.59	20.49	50.2	178–185	DMF-Acetone
IVa	Dihydrochloride	$C_{24}H_{28}Cl_2N_6O_6S_4$	41.43	41.39	...	...	...	...	...	...	...	...	95.6	220–250[a]	Iso-PrOH
V	2-(N-Methylpiperazinomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide	$C_{15}H_{20}N_4O_3S_2$	48.89	48.79	5.47	5.76	...	...	...	...	...	...	60.0	166–173	DMF-50-50 Iso-PrOH-Pet. Ether
Va	Dihydrochloride	$C_{15}H_{22}Cl_2N_4O_3S_2$	40.81	40.75	5.02	5.21	16.07	15.68	12.69	12.39	...	...	83.5	230–238[a]	Iso-PrOH-H_2O
VI	2-(Diethylaminomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide	$C_{14}H_{19}N_3O_3S_2$	49.24	48.64	5.61	5.50	...	...	...	...	18.78	18.96	56.8	205–208	Aqueous HCl-NaOH
VIa	Hydrochloride	$C_{14}H_{20}ClN_3O_3S_2$	44.49	44.29	5.33	5.35	9.38	9.24	11.12	11.22	16.97	17.12	57.7	161–163	Acetone-H_2O
VIb	Acetyl derivative	$C_{16}H_{21}N_3O_4S_2$	50.11	49.73	...	...	...	...	10.96	11.09	...	...	60.5	155–160	Acetone-H_2O
VII	2-(Dimethylaminomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide	$C_{12}H_{15}N_3O_3S_2$	45.99	46.09	4.82	5.00	...	...	13.41	13.50	20.46	20.05	83.8	202–205	MeOH
VIIa	Hydrochloride	$C_{12}H_{16}ClN_3O_3S_2$	41.19	41.08	4.61	4.83	10.14	10.08	12.01	11.90	18.33	18.38	91.5	209–220[a]	EtOH-H_2O

[a] Decomposed slowly over range indicated. [b] DMF, Dimethylformamide. [c] DMS, Dimethyl sulfoxide.

analytical data. By reference to Table I, it can be seen that compounds I, Ia, Ib, II, IIa, III, IIIa, V, Va, VI, VIa, VIb, VII, and VIIa would all have the basic type structure projected as general formula B.[2] Compound IIb is an example of general formula C and compounds IV and IVa represent examples of general formula D.[2]

Samples of representative types of compounds were submitted to the various testing groups to screen for any biological properties that they might possess. The types of activity tested for include: antibacterial and antifungal properties (*in vitro* and *in vivo*), general physiological properties, hypoglycemia, and activity against various parasite organisms and viruses. The results of these biological screening tests will be discussed under the Experimental part of this report.

EXPERIMENTAL DESIGN

The synthesis of the Mannich bases of N-(2-thiazolyl)-1-phenol-4-sulfonamide was carried out by condensing the phenolsulfonamide with the requisite molar proportions of formaldehyde solution and the desired secondary amine in a suitable solvent system (methanol was generally used) using slight modifications of the usual procedures for conducting Mannich reactions of phenols (4), which are described more completely under Experimental (*cf.* procedures A, B, and C). The proposed structures B, C, and D appear to be consistent with their infrared and ultraviolet spectra. For example, in compound types B and C, the infrared hydroxyl band is shifted to above 3 μ for the free Mannich base and is at the normal position of a free hydroxyl group in the hydrochlorides. This phenomenon might be explained by the occurrence of hydrogen bonding in this portion of the molecule.

When a hydrochloride is formed the free electrons on the nitrogen participate and are not free for bonding with the phenolic hydrogen, and the hydroxyl band returns to its normal position. If the Mannich substitution was at any position in the molecule other than *ortho* to the hydroxyl group the above phenomena would probably not occur.

The preparation of the hydrochloride salts (Ia, IIa, IIIa, IVa, Va, VIa, VIIa compounds) from the free bases of the Mannich base derivatives of the phenolsulfonamides was accomplished generally by treating the free base in a suitable solvent system (e. g., acetone, isopropanol, water, or aqueous acetone or isopropanol) with the requisite quantity of hydrogen chloride, either in the form of concentrated hydrochloric acid or hydrogen chloride gas. See procedure D of the Experimental for an example.

The synthesis of the acetyl derivatives (compounds Ib and VIb) of the phenolsulfonamide Mannich bases were effected by treating the compounds with the requisite quantity of acetic anhydride in the presence of pyridine in a suitable solvent system (acetone was used generally). The method used is a modification of many methods found in the literature, examples being the sulfonamide acetylation procedures used by Denton and Quinones (3) and Hoffer (5); see procedure E of the Experimental for a more complete description of the method.

Regarding the proposed structures for the mono-acetyl derivatives (Ib and VIb) of 2-(4-morpholinomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide (I) and 2-(diethylaminomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide (VI), some structural studies were carried out with the aid of infrared spectral analyses in an attempt to determine which of the two available sites for substitution, namely, the 1-phenol hydroxyl or the N^1-hydrogen, the mono-acetylation occurred. All evidence obtained in these studies gives strong evidence that the acetyl group is associated mainly with the 1-phenol position. The infrared curve of the mono-acetylated compounds (Ib and VIb) have C=O maxima at 5.70 μ and the ester peaks at 8.35 μ which is characteristic of phenolic or enolic acetyl ester derivatives. Furthermore, if the acetylation occurred at the N^1 position of the sulfonamide, there should be a maximum at 6.0 μ position of the curves but in all cases it is lacking.

EXPERIMENTAL

Synthesis of the Mannich Bases

Procedure A.—In general, the mono-Mannich bases of the phenolsulfonamides described by structural formula B were synthesized in a manner exemplified by the preparation of compound I.

2-(4-Morpholinomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide, I.—To 1 L. of methanol was added 256 Gm. (1 mole) of N-(2-thiazolyl)-1-phenol-4-sulfonamide and 95.7 Gm. (1.1 mole) of morpholine. On the mixing of these reagents, there was an exothermic reaction and a precipitation of a solid. Eighty-two milliliters (1.1 mole) of 37% formaldehyde solution was now added, the total mixture was stirred at room temperature until complete solution was attained. Finally a solid condensation product appeared. The mixture was heated on a water bath to about 50° for ten minutes. On cooling, more solid separated from the clear, orange liquid. The solid was filtered, suspended in 400 ml. of dimethylformamide, and the suspension was warmed on the steam bath until complete solution was obtained. To the solution was added 500 ml. of methanol and 1,000 ml. of water with stirring and the mixture was placed in the chillroom. Two more crops of product crystallized from the mother liquor and were collected. The crops were combined and dried in an oven at 56°; total yield, 254 Gm. (71.7%). The product melted (decompn.) 194 to 197°.

Anal.—Calcd. for $C_{14}H_{17}N_3O_4S_2$: C, 47.31; H, 4.82; N, 11.82; S, 18.04; mol. wt., 355.4. Found: C, 47.17; H, 5.01; N, 11.41; S, 18.0; mol. wt. by Menzies-Wright method, 350.

Procedure B.—This procedure illustrates the preparation of a di-Mannich base phenolsulfonamide described by structural formula C and exemplified by the preparation of compound IIb.

2,6 - Di - (piperidinomethyl) - N - (2 - thiazolyl)-

[2] The structures assigned to the Mannich bases reported in this paper, represented by general formulas B, C, and D, appear to be acceptable on the basis of microanalytical data, molecular weight determinations, ultraviolet and infrared studies, and an application of the classical rules of substitution and orientation.

1 - phenol - 4 - sulfonamide dihydrochloride, IIb.—Starting with 51.4 Gm. (0.2 mole) of N-(2-thiazolyl)-1-phenol-4-sulfonamide, 17.1 Gm. (0.2 mole) of piperidine, and 18 Gm. (0.2 mole) of 37% formaldehyde solution in 200 ml. of methanol, a batch of the 2-(piperidinomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide was prepared in essentially the same manner as described above. The crude product, about 70 Gm., was then suspended in dimethylformamide. To the resulting suspension, was added an excess (42.5 Gm., 0.5 mole) of piperidine and 37% formaldehyde solution (45 Gm., 0.5 mole). The resulting reaction mixture was stirred and heated on the steam bath for four hours. At this time the mixture was poured into about 750 ml. of water. The precipitate which formed was aged in the chillroom overnight, filtered, washed with water, and dried; weight of product, 15 Gm. (14.6% yield). More product was in the mother liquor but was not worked up. The crude bis-Mannich base was purified by dissolving it in hot chloroform (*ca.* 50 ml.) and filtering the solution. To the clear filtrate was added 500 ml. of petroleum ether causing the product to separate from the solution. The resulting precipitate was aged in a chillroom and filtered. More product was obtained by concentration of the filtrates; yield, 12.5 Gm. (11.8%).

Anal.–Calcd. for $C_{21}H_{30}N_4O_5S_2$: N, 12.43; S, 14.23. Found: N, 12.02; S, 14.29.

The 2,6-di-(piperidinomethyl)-N-(2-thiazolyl)-1-phenolsulfonamide (10 Gm.; 0.019 mole) was converted to the corresponding dihydrochloride by treating with an excess of concentrated hydrochloric acid in about 100 ml. of isopropanol. The product was isolated by distilling off the solvents *in vacuo*. The residue was purified by dissolving it in hot dimethylformamide (*ca.* 20 ml.) and precipitating the product by addition of an excess of a 50:50 mixture of petroleum ether and isopropanol (*ca.* 200 ml.). The product was aged in the chillroom for two hours, filtered, and dried *in vacuo*; yield, 8 Gm. (68% conversion). It melted (decompn.) in the range of 184–191°.

Anal.–Calcd. for $C_{21}H_{32}Cl_2N_4O_5S_2$: C, 48.17; H, 6.16; Cl, 13.54; N, 10.70; S, 12.25; mol. wt., 523.5. Found: C, 48.33; H, 6.96; Cl, 13.68; N, 10.72; S, 11.78; mol. wt., 540 (determined by differential vapor pressure thermistor method).[1]

Procedure C.—This procedure illustrates the preparation of a bis-phenolsulfonamide Mannich base of the type described by structural formula D and exemplified by the preparation of compound IV.

N,N' - Di - [N -(2 - thiazolyl) - 5 - sulfonamido - 2-hydroxybenzyl]-piperazine, IV.—To 400 ml. of methanol was added 104 Gm. (0.4 mole) of N-(2-thiazolyl)-1-phenol-4-sulfonamide, 38.8 Gm. (0.2 mole) of piperazine hexahydrate, and 36 Gm. (0.4 mole) of 37% formaldehyde to form complete solution. After the solution had been stirred and heated on a steam bath for about five minutes, a cloudiness appeared and an oily product began to separate from the solution. The mixture was permitted to stir one hour and then was cooled to room temperature. The crude product was dissolved in 600 ml. of dimethylformamide with the aid of gentle warming of the steam bath. The solution was treated with a large excess (4,800 ml.) of acetone. The mixture was stirred well and permitted to stand in the chillroom overnight. The crystals which separated were filtered, washed with two 100-ml. portions of acetone, and dried. Several more crops of crystals separated from the mother liquor on standing. These were filtered and dried; total yield, 62.5 Gm. (50.2%); m. p. 178–185° (decompn.).

Anal.—Calcd. for $C_{24}H_{28}N_6O_6S_4$: C, 46.29; H, 4.21; N, 13.50; S, 20.59. Found: C, 45.84; H, 4.48; N, 13.15; S, 20.49.

Synthesis of the Hydrochloride Salts

Procedure D.—The preparation of compound IIA represents a typical conversion of the free base of a Mannich derivative of a phenolsulfonamide to the corresponding hydrochloride salt.

2 - (Piperidinomethyl) - N - (2 - thiazolyl) - 1-phenol-4-sulfonamide hydrochloride, IIa.—To 1 Gm. (0.0028 mole) of 2-(piperidinomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide was added 20 ml. of isopropanol. Into the resulting mixture was bubbled hydrogen chloride gas. At first the solid dissolved in the solution but then the hydrochloride began to separate. The flask was chilled well in an ice bath to permit complete separation of the hydrochloride salt. It was filtered, washed a few times with small portions of isopropanol, and dried. The hygroscopic product was recrystallized by dissolving it in a small portion of methanol-ethanol and precipitating with an excess of anhydrous ether. The mixture was permitted to stand in the chillroom overnight. The product which separated from the mixture was filtered and dried; yield, 0.54 Gm. (50%); m. p. 185–189° (decompn.).

Anal.—Calcd. for $C_{15}H_{20}ClN_3O_3S_2$: C, 46.20; H, 5.17; Cl, 9.09; N, 10.78; S, 16.45. Found: C, 46.23; H, 5.38; Cl, 8.83; N, 10.59; S, 16.23.

Synthesis of the Acetyl Derivatives

Procedure E.—The preparation of compound Ib represents an example of a procedure for preparing a mono-acetyl derivative of these Mannich phenolsulfonamides.

Acetyl Derivative of 2-(4-Morpholinomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide, Ib.—To 3.55 Gm. (0.01 mole) of 2-(4-morpholinomethyl)-N-(2-thiazolyl-1-phenol-4-sulfonamide was added 1.47 ml. (0.014 mole) of acetic anhydride, 1.6 ml. (0.02 mole) of pyridine, and 10 ml. of acetone. The mixture was stirred until nearly all of the solid was in solution (about five minutes) and then the mixture was heated to about 50° under reflux. At this time some precipitation occurred. The mixture was permitted to stand at 40° for thirty minutes and was then placed in the chillroom. To the mixture was then added 10 ml. of an ice cold 3% ammonia solution, with a few ice chips. The precipitate was filtered and washed with 10 ml. of 1% ammonia solution, followed by 10 ml. of water, and then 3 ml. of ethanol. The product was dried at 56° for one hour; yield, *ca.* 1 Gm. (25%); m. p. 165–167° (decompn.).

Anal.—Calcd. for $C_{16}H_{19}N_3O_4S_2$: C, 48.35; H, 4.82; N, 10.57; S, 16.13; acetyl value, 10.8; mol. wt., 397.5. Found: C, 48.89; H, 5.19; N, 10.37; S, 15.95; acetyl value, 10.7; mol. wt. by Menzies-Wright method, 403.

[1] This method was presented by A. Wilson, *et al.*, at the American Chemical Society Meeting-in-Miniature, Seton Hall, East Orange, N. J., January 1959.

TABLE II.—*In Vitro* ANTIBACTERIAL AND ANTIFUNGAL STUDIES[a,b]
Minimal Inhibitory Concn. γ/ml.

No.	Compound	*Trichophyton mentagrophytes* E-11	*Sarcina lutea* ATCC 9341	*Bacillus subtilis* ATCC 6633	*Proteus vulgaris* STCC 8427	*Salmonella gallinarum* Led. An. Ind. 604
I	2-(4-Morpholinomethyl)-N-(2-thiazolyl)-1-phenol-sulfonamide	...[c]	250 62[d]	...	125	125[d]
Ia	Hydrochloride	...	250	250	250	250
Ib	Acetyl derivative	...	...	...	...	...
II	2-(Piperidinomethyl)-N-(2-thiazolyl)-1-phenol-sulfonamide	...	250 62[d]	...	250 62[d]	...
IIa	Hydrochloride	...	125	...	125	...
IIb	2,6-Bis compound dihydrochloride	...	250 62	250	250	...
III	2-(Pyrrolidylmethyl)-N-(2-thiazolyl)-1-phenol-sulfonamide	...	250	...	...	...
IIIa	Hydrochloride	...	...	...	...	...
IV	N,N'-di-[N-(2-thiazolyl)-5-sulfonamido-2-hydroxybenzyl] piperazine	62 8[d]	...	250	...	...
IVa	Dihydrochloride	...	...	...	...	...
V	2-(N-Methylpiperazinomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide	...	...	...	125[d]	...
Va	Dihydrochloride	...	250	...	125[c]	...
VI	2-(Diethylaminomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide	...	250 62[d]	...	...	...
VIa	Hydrochloride	...	...	...	...	...
VIb	Acetyl derivative	250	...	...	...	...
VII	2-(Dimethylaminomethyl)-N-(2-thiazolyl)-1-phenol-4-sulfonamide	250	125[d]	...	...	...
VIIa	Hydrochloride	...	...	...	...	...

[a] Test bacteria against which the compounds showed no activity include: *Mycobacteriums megmatis, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli.* [b] Test fungi against which the compounds showed no activity include: *Candida albicans, Saccharomyces carlsbergensis, Mucor ramannianus, Fusarium episphaeria, Hormodendrum cladosporoides, Microsporum gypseum, Botrytis cinerea, Penicillium digitatus, Myrotheciun verrucaria, Allernaria fasciculata,* and *Aspergillus fumigatis.* [c] Test compound inactive at highest test level used, namely, 250 mcg./ml. [d] Partial inhibition.

BACTERIOLOGICAL STUDIES

Antibacterial and Antifungal *In Vitro* Studies.—Representative types of compounds from this series of Mannich base derivatives of phenolsulfonamides were placed in the *in vitro* antibacterial and antifungal screening program. The special bacteriological media (Mueller-Hinton) for sulfonamides (containing no *p*-aminobenzoic acid) was used for these studies. As the data given in Table II indicates, the antibacterial properties of these compounds are not particularly impressive. Some activity was demonstrated by most candidate compounds against at least one or two of four bacteria, namely: *Sarcina lutea* (ATCC 9341), *Bacillus subtilis* (ATCC 6633), *Salmonella gallinarum* (LAI 604)$_h$, and *Proteus vulgaris* (ATCC 8427), from a total of eight test organisms. The minimal inhibitory concentration of these compounds in terms of γ/ml. required, however, was fairly high in each case, indicating a relatively low order of activity in all cases. A study of Table I will show that the candidate compounds showing activity against the greatest number of bacteria (three or four) are I, Ia, and IIb.

Against approximately twelve test fungi used for screening purposes, three of the compounds tested namely: IV, VIb, and VII, demonstrated some activity against one fungus, *Trichophyton mentagrophytes* (Ell). Of these compounds IV exhibited fairly good antifungal activity being inhibitory at 62 γ/ml. and showing partial inhibitance at 8 γ/ml. However this piperazine derivative failed to show any significant activity against the other test fungi. Although not indicated in the table, compound IVa did show partial inhibitory action against *Candida albicans* at 125 γ/ml.

Antibacterial *In Vivo* Studies.—None of the representative candidate compounds tested exhibited any appreciable activity in *in vivo* antibacterial studies.

Virological Studies.—None of the representative candidate compounds tested exhibited any antiviral activity against virus organisms, namely, Columbia S. K. and Influenza A (PR 8).

Pharmacological Studies.—Representative types of the phenolsulfonamide Mannich bases and their derivatives were submitted for preliminary pharmacological, gross observation studies in mice. None of the compounds tested exhibited any interesting symptomatology in these screening tests.

Several of the compounds (I, Ib, II, IV, and Va) prepared in this series were tested for any potential oral hypoglycemia activity. All of these compounds proved to be inactive in this respect.

Parasitological Studies.—With the knowledge that certain piperazines have demonstrated effective action against certain types of parasites, particularly certain species of roundworms, pinworms, hookworms, etc., strongly infecting domestic animals (6), several Mannich bases of this series, in which a piperazine molecule was substituted into the phenolsulfonamide nucleus, namely, compounds IV, IVa, V, and Va, were submitted for parasitological screening. These compounds were tested against *Schistosoma mansoni* (blood flukes) in mice at 100 mg./Kg., orally, twice daily for five days, and found to be inactive.

The same compounds were also inactive against *Nematospiroides dubius* (intestinal helminths) in mice.

SUMMARY

1. The synthesis of eight Mannich bases, derived from N-(2-thiazolyl)-1-phenol-4-sulfonamide and related hydrochlorides and acetyl derivatives, has been described.

2. Representative candidate compounds of this series were tested. With the exception of some slight antibacterial and antifungal activity (*in vitro*) these compounds failed to demonstrate any biological properties of significant interest.

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Rate of Release of Medicaments from Ointment Bases Containing Drugs in Suspension

By TAKERU HIGUCHI

An equation relating the rate of release of solid drugs suspended in ointment bases into perfect sinks is derived. The final expression is found to be surprisingly simple and convenient.

IN AN EARLIER publication (1) an equation was given relating the amount and rate of release of materials suspended in an ointment base to time and variables of the system

$$Q = (2A - C_s)\sqrt{\frac{Dt}{1 + \frac{2(A - C_s)}{C_s}}}$$

where Q = the amount absorbed at time t per unit area of exposure, A = the concentration of drug expressed in units/cm.^{3}, C_s = the solubility of the drug as units/cm.^{3} in the external phase of the ointment, and D = the diffusion constant of the drug molecule in the external phase.

The present communication is concerned with the theoretical derivation of this relationship. Normally, diffusional calculations are extremely complex and lead to unwieldy expressions. In the present instance, despite the obvious complexity of the type of system involved, the equation comes out rather simply and in a useful form.

The equation is derived for a system described as follows: (*a*) the suspended drug is in a fine state such that the particles are much smaller in diameter than the thickness of the applied layer; (*b*) the amount of drug, A, present per unit volume is substantially greater than C_s, the solubility of the drug per unit volume of the vehicle; (*c*) the surface to which the drug ointment is applied is immiscible with respect to the ointment and constitutes a perfect sink for the released drug.

All of the above conditions are evidently self apparent, except possibly for the reference to a perfect sink. In the publication cited above it was pointed out that the rate of absorption of drug from an ointment could be effectively limited by any one of the three processes involved: (*a*) drug clearance below the "barrier layer," (*b*) passage through the "barrier layer," or (*c*) release by the ointment itself.

It was shown that in the cases where either of the first two processes were rate limiting, only the thermodynamic activity of the drug in the base was important. If the last process is of paramount importance, then we are tacitly assuming that the surface to which the ointment is applied acts as a perfect sink.

DERIVATION OF THE EQUATION

For such a system we can draw a concentration profile which may exist after the lapse of finite time after application of the ointment (Fig. 1). The solid line in the diagram would essentially represent the concentration gradient existing after time, t, in the ointment layer normal to the absorbing surface. The total drug concentration, as indicated in the drawing, would be expected to show a more or less sharp discontinuity at distance h from the surface, none of the suspended phase dissolving until the environmental concentration drops below C_s. The sharpness of the break will be largely a func-

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tion of the fineness and the state of dispersion of the solid phase. For the distance, h, above the absorbing surface the concentration gradient would be essentially constant, provided $A \gg C_s$. The linearity of the gradient over this distance follows under these conditions from Fick's first law. The shift in the profile after additional interval of Δt is shown as a dotted line in the diagram, corresponding to the extension of the zone of partial depletion by the distance Δh. It is evident, furthermore, that at time, t, the amount of material absorbed or, equivalently, depleted from the ointment corresponds to the shaded area in the diagram.

It can be seen, based on the above diagram, that dQ, the amount additionally depleted by further movement of the front by dh, is related to other constants

$$dQ = A\,dh - {}^1/_2 C_s\,dh$$

But according to Fick's law

$$dQ/dt = DC_s/h$$

or

$$A\frac{dh}{dt} - \frac{1}{2}C_s\frac{dh}{dt} = \frac{DC_s}{h}$$

$$\frac{(2A - C_s)h}{2DC_s}\,dh = dt$$

Integrating both sides, we get

$$t = \frac{h^2}{4DC_s}(2A - C_s) + K$$

where K is the integration constant.

It is apparent that $K = 0$ for t measured from zero, or

$$t = \frac{h^2}{4DC_s}(2A - C_s)$$

and

$$h = 2\sqrt{\frac{DtC_s}{2A - C_s}}$$

From the diagram it is apparent that the amount of depletion Q, at time, t, is

$$Q = hA - \frac{hC_s}{2}$$

Substituting for h from above we get

$$Q = \left(A - \frac{C_s}{2}\right)\,2\sqrt{\frac{DtC_s}{2A - C_s}} = \sqrt{Dt(2A - C_s)C_s}$$

These relationships will be essentially valid for all time less than that corresponding to complete depletion of the suspended phase. The initial lag time corresponding to the time necessary for establishment of a quasi-stationary state conforming to the equations would generally be less than

$$L = \frac{[n\alpha]^2}{6D}$$

where α is the mean distance between the suspended particles and n is of the order of 2 or 3. Since the interparticulate distance is assumed to be extremely small for the model system relative to the layer thickness, the lag time is expected to be very short in comparison to the total depletion period.

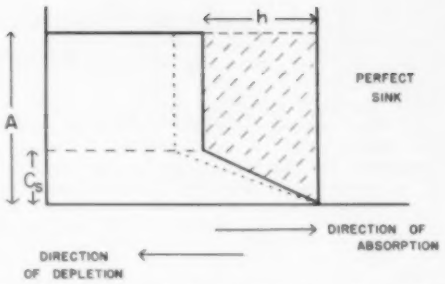

Fig. 1.—Theoretical concentration profile existing in an ointment containing suspended drug and in contact with a perfect sink.

Differentiating with respect to time, we obtain the instantaneous rate of absorption at time t

$$\frac{dQ}{dt} = \frac{1}{2}\sqrt{\frac{D(2A - C_s)C_s}{t}}$$

For the common case of $C_s \ll A$, the relationship simplifies to

$$Q = \sqrt{2ADC_s t}$$

and

$$\frac{dQ}{dt} = \frac{ADC_s}{2t}$$

These relationships are identical with or equivalent forms of the earlier expressions.

According to these remarkably simple relationships, remarkable in view of the complexity of the situation treated, the amount of drug released from such suspension-type ointment ($C_s \ll A$) is proportional to the square root of the amount of drug per unit volume, diffusion constant, drug solubility, and time. It is of interest to note that intuitively one might expect a direct relationship with concentration, but this is not the case.

It is evident that we can regulate the rate of release of drugs from such preparations by controlling A, D, and C_s. If partly aqueous base is employed, C_s can be varied, for example, by changing the effective pH of the vehicle for insoluble acidic and basic drugs. Or it can be altered by addition of complexing agent or cosolvent. D, the diffusion coefficient, is inversely proportional to the microscopic viscosity of the vehicle and may be varied in this manner. A, the drug concentration, is, of course, susceptible to wide variation.

There are a number of other similar situations which have been solved dealing with diffusional flow where all of the gradient is in the applied phase. We have worked out cases involving both emulsion-type ointments and ointments containing solid fillers such as zinc oxide. Nearly exact mathematical solutions to the behavior of these systems as drug sources are presently available.

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Chemical Studies of the Leaves of *Withania somnifera*

By N. S. DHALLA, M. S. SASTRY, and C. L. MALHOTRA

Besides the preliminary investigations, a new crystalline compound named withanone (m. p. 263°) has been isolated from the leaves of *Withania somnifera*. Withanone is found to contain lactone and steroidal rings in the molecule. Somnitol, glucose, and a large quantity of inorganic salts have also been separated. The total alkaloids, on subjecting to paper chromatography, indicate five alkaloidal bands. The presence of cystine, glycine, glutamic acid, α-alanine, proline, and tryptophan is also reported.

WITHANIA SOMNIFERA Dunal (*Solanaceae*) is indigenous to India and is used in the ayurvedic system of medicine as a general tonic and in the treatment of various nervous disorders. The leaves are applied locally to tumors and to tuberculous glands. A fomentation of the leaves is used to cure sore eyes, ulcers, and swellings. They are also used as a hypnotic and an anthelmentic (1–3).

The earlier workers (4–6) had used the whole plant for chemical examination and reported the isolation of hentriacontane, fatty acids, ipuranol, withaniol, somnirol, somnitol, essential oils, and a number of alkaloids. Recently Srivastava, *et al.* (7), have described a method for the estimation of the total alkaloids of *Withania somnifera*. The presence of various amino acids in the berries of the plant has been reported by Atal (8). Owing to the isolation of a therapeutically active antibiotic substance (9) from the leaves of the plant, it was considered necessary to work out the chemical composition of the leaves.

EXPERIMENTAL

The plant material was collected in the Delhi area, properly identified, and the leaves were dried under shade. The powdered leaves were successively extracted with different solvents and the percentage yield of the extracts were as follows: petroleum ether (40–60°) 1.32; ether 1.05; chloroform 3.36, and alcohol 10.44%. The defatted material yielded 1.20, 9.65, and 0.28% extractives when exhausted in succession with benzene, methanol, and acetone, respectively, in a Soxhlet extractor. Alkaloids, glycosides, reducing sugars, and inorganic salts were found to be present. The total ash (7.4%) and the acid-insoluble ash (1.8%) contained chloride, sulfate, iron, potassium, and sodium.

Isolation of Withanone.—A 5-Kg. portion of the powdered drug was extracted with boiling alcohol and the combined extracts were concentrated under reduced pressure to a syrupy mass. It was extracted with ether and the ether-soluble fraction (*E*) was worked out separately. The ether-insoluble residue was freed from dissolved ether and exhausted with water to get the water-soluble fraction (*W*). The water-insoluble mass was treated with cold 90% alcohol and the alcohol-insoluble residue was dried, taken in chloroform, and filtered. The filtrate was decolorized with animal charcoal and evaporated to get a white substance which, on repeated crystallization from absolute alcohol, yielded fine, rod-shaped crystals (yield 0.102%) melting at 263°.

The crystalline material was soluble in methanol, chloroform, and hot alcohol; and insoluble in other common organic solvents, dilute acids, and dilute alkalies. It charred on heating and gave a pungent odor on fusion with potassium hydroxide. It gave a crimson red color with concentrated sulfuric acid. In Moleschott's reaction the compound gave an orange-red color, while a yellow color changing to brown was developed in Hirschsohn's reaction. Liebermann-Burchard and Salkowski tests were also positive, confirming the presence of a steroidal ring in the molecule. It did not decolorize bromine solution and gave a negative Legal reaction. The empirical formula $C_{28}H_{32}O_6$ is based on the analysis of carbon and hydrogen content.

Anal.—Found: C, 71.5; H, 8.1. It gives maximum absorption in the ultraviolet at 245 mμ. In the infrared studies the compound shows well-defined bands due to the ketonic and hydroxyl groups. On boiling with alcoholic sodium hydroxide, the compound dissolves, giving a red solution which on acidifying deposits a yellow flocculant precipitate melting at 185°.

Isolation of Somnitol.—The ether-soluble fraction (*E*) was evaporated to dryness and the residue was redissolved in chloroform. The chloroform-soluble fraction was decolorized and the residue obtained after evaporation was crystallized from absolute alcohol when more of the compound (m. p. 263°) described above was separated. The mother liquor was diluted with water to get a turbid solution and allowed to cool in a refrigerator when a faintly colored substance was deposited. This, on crystallization from dilute alcohol, yielded white flakes melting at 248–250° (yield 0.003%). It was identified as somnitol, $C_{28}H_{46}O_7$, from its properties and elemental analysis.

Isolation of Glucose and Inorganic Salts.—The water-soluble fraction (*W*) obtained earlier was boiled with animal charcoal and the filtrate was evaporated to dryness. The mass was dispersed in boiling methanol and filtered while hot. The filtrate was concentrated and allowed to remain at

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room temperature for a number of days, when a small quantity of crystalline material was found adhering to the sides of the container. This was found to be a reducing sugar having a sweet taste. From the characteristics and the osazone derivative, it was identified as glucose. Methanol-insoluble material (yield 0.38%) was found to be inorganic in nature and contained chloride, sulfate, nitrate, sodium, and potassium.

Alkaloids.—The total alkaloids (yield 0.09%) were isolated by extraction with 2% sulfuric acid, precipitation with Dragendorff's reagent, and decomposition with freshly prepared silver carbonate. The alkaloids were subjected to paper chromatography using a mixture of *n*-butanol, acetic acid, water (4:1:5) as solvent. When the chromatogram was viewed under ultraviolet light, two bands with green fluorescence having R_f values 0.31 and 0.38 were observed. The chromatogram, on spraying with Dragendorff's reagent (10), revealed the presence of three more bands having R_f values 0.50, 0.58, and 0.80, in addition to the above two bands.

Amino Acids.—The powdered leaves were freed from chlorophyll and other coloring matter and extracted with 80% alcohol. The extract was evaporated to dryness and redissolved in 95% alcohol containing 0.5% hydrochloric acid. This was repeatedly evaporated until free from mineral acid and finally was dispersed in water. Paper chromatographic analysis using a mixture of phenol, isopropanol, water (70 : 5 : 25) in an atmosphere of 0.1% ammonia, and a mixture of *n*-butanol, acetic acid, water (4:1:5) as solvents showed, on spraying with ninhydrin reagent, the presence of cystine, glycine, glutamic acid, α-alanine, proline, and tryptophan.

DISCUSSION

A new crystalline compound withanone, m. p. 263°, has been isolated from the leaves of *Withania somnifera*. It was found to exhibit a number of positive color reactions typical of steroids. The solubility of the compound in hot alcoholic sodium hydroxide and the separation of an acid on acidification indicated the presence of a lactone ring, which was further confirmed by the well defined band in infrared absorption spectrum at 5.9 μ due to C=O group. A negative Legal reaction and the insolubility of the compound in boiling 10% sodium hydroxide solution showed the saturated nature of the lactone. The absence of unsaturation was also found from its behavior towards bromine solution. The compound did not show any absorption in the ultraviolet near 220 mμ and this excluded the possibility of the presence of an α,β-unsaturated lactone in the molecule. Further work regarding the constitution of withanone is in progress.

SUMMARY

1. Chemical examination of the leaves of *Withania somnifera* Dunal showed the presence of alkaloids, glycosides, reducing sugars, and inorganic salts.

2. A new crystalline compound withanone (m. p. 263°) was isolated from the alcoholic extract. The compound was found to contain lactone and steroidal rings in the molecule.

3. Somnitol and glucose were also separated, in addition to inorganic salts containing chloride, sulfate, nitrate, sodium, and potassium.

4. Paper chromatography of total alkaloids showed the presence of five bands having R_f values 0.31, 0.38, 0.50, 0.58, and 0.80.

5. The presence of cystine, glycine, glutamic acid, α-alanine, proline, and tryptophan was detected.

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Effects of Ethylenediaminetetraacetic Acid (EDTA) on Prothrombin Consumption

By STEVEN G. BJAASTAD† and NATHAN A. HALL

Whole human blood was treated with varying concentrations of ethylenediaminetetraacetic acid to determine the effect upon prothrombin consumption. The prothrombin consumption test was combined with the Dreskin two-stage prothrombin test to determine residual prothrombin as a function of incubation time. Ethylenediaminetetraacetic acid in high concentrations inhibited prothrombin consumption; in one concentration prothrombin consumption was accelerated, and in the lower concentrations no effect was observed. These data indicate that EDTA has a diphasic action upon the utilization of prothrombin as a part of its diphasic action in whole blood coagulation.

ETHYLENEDIAMINETETRAACETIC acid (EDTA) is a strong chelating agent of divalent alkaline earth metal ions and has been recommended as an anticoagulant for blood (1). The logarithm of the stability constant of the EDTA-calcium complex in the pH range 7–8, 3.51, compares favorably with the logarithms of the stability constants for the calcium complexes of the classical anticoagulant acids, citric and oxalic (3.2 and 3.0, respectively) (2).

EDTA has been shown to exhibit a diphasic action on the coagulation of whole blood; thus high concentrations of EDTA act as an anticoagulant and low concentrations as an accelerator of coagulation. The maximum acceleration of coagulation has been reported at a concentration of about 1×10^{-4} *M* (3). The study reported here was undertaken to examine the effects of EDTA upon one of the stages of coagulation, the conversion of prothrombin to thrombin.

EXPERIMENTAL

Whole human blood was used as the test material. It was obtained from volunteers who had fasted for at least four hours prior to sampling.

Fibrinogen solutions contained 720 mg. of Armour bovine fibrinogen dissolved in sufficient lactated Ringer's solution to make 100 ml. This solution was frozen in 3.5-ml. lots and stored at −10° until used.

The disodium ethylenediaminetetraacetate (EDTA·Na_2) was reagent grade (ethylenedinitrilo)-tetraacetic acid disodium salt (Eastman). Solutions for addition to blood were prepared by diluting the desired quantity of 0.025 *M* EDTA·Na_2 stock solution with 2 ml. of 0.25 *M* imidazole buffer (4), pH 7.25, and adding sufficient 0.15 *M* sodium chloride solution to make 10 ml. The resulting solution, buffered to pH 7.25 with 0.05 *M* imidazole, had a calculated ionic strength of 0.15 and contained sufficient EDTA·Na_2 to give the desired blood concentration when 0.1 ml. was diluted with 1.0 ml. of blood.

All syringes, needles, and glassware, except incubation tubes, were silicone-coated with Siliclad[1] which was removed and freshly reapplied before reuse.

The prothrombin utilization test was a modification of the method of Graham, Langdell, and Brinkhous (5) in which the two-stage prothrombin determination of Dreskin (6) was used. Three 10-ml. samples of venous blood were taken from each subject by a multiple syringe technique. The first sample taken was used as the source of red cells and platelets for the two-stage prothrombin determination and treated by the addition of 1.0 ml. of 0.11 *M* sodium oxalate to 9.0 ml. of blood. The cells and platelets were washed three times before use with 0.15 *M* sodium chloride. After the first sample of blood was withdrawn, a new syringe was attached to the needle which remained in the vein, and the second 10-ml. sample was taken. The third sample was taken through the same needle in yet another syringe. The second and third, the test samples, were processed simultaneously, with the second sample being treated with EDTA·Na_2 and the third sample used as the control.

Immediately after removal from the vein, 2.0 ml. of the test blood was placed in a centrifuge tube containing 0.3 ml. of 0.05 *M* EDTA·Na_2 solution and 0.1 ml. of imidazole buffer. The remaining 8 ml. of blood was distributed equally into eight test tubes (10 × 75 mm.) suspended in a water bath at 37.5 ± 0.5°. The tubes in the water bath contained either 0.1 ml. of 0.05 *M* imidazole buffer for the control samples or 0.1 ml. of EDTA-buffer for the test samples. The timing of the incubation period was started when the initial 2.0 ml. of blood was placed in the centrifuge tube, and thrombin generation was stopped at the end of the incubation periods by addition of 0.05 *M* EDTA·Na_2 (0.15 ml.).

Residual prothrombin was determined by the Dreskin two-stage prothrombin test (6). A 0.12-ml. quantity of sample plasma or serum was mixed with 0.3 ml. of washed cells, and 9.68 ml. of barbital buffered 0.02 *M* calcium chloride solution was added. This mixture was incubated in a water bath at 37.5 ± 0.5°. At six, ten, and fourteen minutes after addition of the calcium chloride solution, 0.2-

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[1] Trademark, Clay-Adams, Inc., New York, N. Y.

TABLE I.—PROTHROMBIN REMAINING AT SPECIFIED INCUBATION TIMES[a]

Molar EDTA Concn. $\times 10^{-3}$	No.	Incubation Time, min. 0	20	40	80	120
Control	27	100	48.8 ± 3.0	19.6 ± 1.3	12.4 ± 0.7	10.5 ± 0.7
2.00	3	100	93.8 ± 2.0	84.0 ± 5.1	70.0 ± 5.3	64.5 ± 2.1
1.75	3	100	79.5 ± 4.7	57.3 ± 4.3	46.7 ± 3.8	41.9 ± 2.9
1.00	3	100	63.4 ± 2.3	32.9 ± 2.2	22.8 ± 5.2	21.3 ± 5.6
0.50	3	100	46.2 ± 12.8	25.2 ± 2.8	17.5 ± 2.1	15.5 ± 2.4
0.25	3	100	35.9 ± 5.4	17.2 ± 0.9	14.1 ± 2.1	13.7 ± 2.2
0.10	3	100	50.2 ± 8.3	22.6 ± 3.6	14.2 ± 1.8	12.7 ± 1.8
0.01	3	100	59.7 ± 8.6	25.7 ± 4.2	13.8 ± 1.8	11.1 ± 0.9

[a] Figures are average per cent residual prothrombin ± standard error.

ml. samples were withdrawn and mixed with 0.2 ml. of fibrinogen solution. The time required for the appearance of a fibrin network at the boundary between the glass of the test tube and the solution was taken as the clotting end point, and time of the sample having the shortest clotting time of the three was accepted as the measure of the prothrombin content.

Prothrombin concentrations were expressed as the per cent remaining after incubation and were calculated by the following formula:

$$\% \text{ residual prothrombin} = 100 \times \frac{\text{prothrombin time of sample not incubated}}{\text{prothrombin time of incubated sample}}$$

Seven different concentrations of EDTA were used, ranging from 2.0×10^{-3} *M* to 1.0×10^{-5} *M* in whole blood. Three samples were run at each concentration level, along with a simultaneous control. The amount of prothrombin remaining in the samples from various incubation times was recorded in Table I.

DISCUSSION

Because freshly drawn blood samples were essential to the study, pooled blood could not be used; therefore, considerable variation in calcium levels would be expected and is shown by the relatively high standard errors. In spite of this drawback, certain trends are evident from the results. At high concentrations, greater than 0.50×10^{-3} *M* EDTA significantly inhibits the utilization of prothrombin in the initial twenty minutes of coagulation. At 0.25×10^{-3} *M* EDTA concentration, there seems to have been an acceleration of the initial stages of coagulation. Statistical analysis of the difference between the per cent prothrombin remaining in this sample and the control samples was carried out using the *t* test and the *P* of the difference being due to chance was less than 0.02.

Further reduction of the EDTA concentration returned the initial prothrombin consumption to control levels. The residual prothrombin for the longer incubation periods was higher than control for the EDTA samples in all cases, but in the samples with low EDTA concentrations the difference from the control values was not significant.

Because a blood clot in whole blood usually forms within twenty minutes, the previously reported diphasic effect of EDTA should be observed in the rate of prothrombin consumption within the first twenty minutes of incubation. Although the number of observations is small, this effect is shown by the 0.25×10^{-3} *M* EDTA sample in which, after twenty minutes of incubation, the prothrombin consumed is significantly greater than the control, but not in higher or lower concentrations.

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Effects of Processing on the Rheology of Thixotropic Suspensions

By THOMAS H. SIMON, H. GEORGE DeKAY, and GILBERT S. BANKER

A study was undertaken to evaluate the effects of processing by various types of commercially available dispersion equipment under different conditions of operation on certain rheological parameters of thixotropic suspensions. Two suspending agents, bentonite U. S. P. and Veegum HV were used in the evaluation procedure and four types of dispersion equipment were used, a marine-type impeller mixer, a homogenizer, a colloid mill, and a high shear mixer. The rheological parameters were obtained from flow curves that were automatically plotted by means of a completely instrumented Ferranti-Shirley cone-plate viscometer. The type of dispersion equipment employed and the operating conditions used were related to the rheological parameters of the suspension systems. The rheological parameters evaluated were thixotropic index, yield value, spur value, and plastic viscosity.

LIQUID PREPARATIONS containing insoluble therapeutic agents are extensively used in pharmacy for products designed for both internal administration and external application. Satisfactory suspension systems of this type exist when settling and caking are at a minimum, thus eliminating, as nearly as possible, the danger of nonuniform dosage occurring as the product is administered. This is especially important in light of the increasing trend toward more potent medication. With few exceptions, the nature of the insoluble therapeutic agents incorporated into liquid preparations is such that they do not impart to the system the desired stability characteristics. For this reason it is necessary to include in the formulation of such products some other agent or agents to provide the required stability. Agents capable of producing thixotropic suspensions have found wide acceptability for this purpose. The amount of thixotropic suspending agent normally incorporated into a formula is generally the lowest concentration that will produce the desired stability and consistency characteristics. From a production standpoint, this amount should be determined under normal operating conditions and on equipment currently available in the plant.

In the preparation of suspensions, colloid mills, homogenizers, and high shear mixers are used in conjunction or following simple mixing with a turbine or marine-type impeller. Investigation of the literature indicates that altering the type of dispersion equipment used and the conditions of processing employed, could conceivably alter the physical characteristics of the suspension system involved. Kostenbauder and Martin (1), studying the rheological properties of semisolids, determined that thermal history and previous mechanical treatment affect the rheological properties of non-Newtonian systems. Working with carboxymethylcellulose, deButts, *et al.* (2), showed that the gel strengths of such solutions are materially affected by the rates of shear at which the solutions are prepared. King and Becker (3) correlated the physical stability of calamine lotion prepared with a thixotropic suspending agent to the method of preparation employed. They prepared the calamine lotion with the aid of a colloid mill, a homogenizer, a marine-type impeller mixer, and a mortar and pestle. Maximum stability occurred after the material was processed by the homogenizer.

Physical systems involving non-Newtonian materials are, at best, extremely complex, requiring an evaluation of several rheological parameters to describe certain physical and consistency characteristics. The rheological parameters of suspension systems can be related in many instances to the stability characteristics of the entire system. Foernzler, *et al.* (4), have shown that thixotropic index can serve as a means of measuring suspension stability when related to the sedimentation volume of an insoluble constituent. It is thus possible to evaluate the relative stabilities of several suspensions by examination of this particular rheological parameter. In addition, the rheological parameters yield value, spur value, and plastic

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viscosity also serve to describe certain characteristics of a suspension system.

A knowledge of the relationship between processing with regard to both type of equipment and conditions employed and rheological parameter could suggest to the formulator of a new product the equipment to be utilized which would probably result in the most economical use of material, time, and equipment. In addition, such knowledge might provide the production engineer with a new working variable to modify existing or new products or to locate a source of trouble in production runs that fail to meet specifications.

In a recent article (5), Samyn discussed the effect of mixing intensity and mixing time on the extent of hydration of Veegum HV and on practical scale-up and production problems. He also presented some experimental data to show the relationship between hydration time of the clay and Brookfield viscosity measurements. A survey of the literature has revealed no information, however, on the magnitude of the effect of shear rate on plastic viscosity, yield value, or thixotropy of clay-type suspending agents.

The purpose of this research was to investigate the changes and relative magnitude of changes in the rheological parameters of common clay-type suspending agents produced by varying the type of dispersion equipment used and the operating conditions employed.

EXPERIMENTAL

Suspending Agents.—Two suspending agents, both producing thixotropic vehicles, were used in this study. Bentonite U. S. P.[1] was used as an example of an official product that has wide application and has enjoyed extensive use. Veegum HV[2] was selected as an example of the type of highly purified commercial product whose use is becoming increasingly more extensive. Suspensions of these clays were prepared in concentrations based on those most commonly encountered in practice. The bentonite suspensions were prepared in concentrations of 4, 5, and 6% according to the method outlined in U. S. P. XVI. Veegum HV suspensions were prepared in concentrations of 4 and 5% by the method recommended by the manufacturer (6). Each suspension was prepared in a batch size of 20 L. The large batch size was necessary to allow all processing to be accomplished on a single batch so that any subsequent differences in rheological parameters could be attributed to the processing conditions employed.

Processing Equipment.—Four types of processing equipment were used in this study. Preliminary mixing was accomplished with a marine-type impeller mixer at 1,700 r.p.m. This agitator, referred to as the wall mixer, had a 70-cm. shaft and a 10.5-cm. arc described marine-type impeller driven by a one-quarter horsepower motor mounted vertically on a movable frame. The mixing vessel utilized had a diameter of 36 cm. and was fitted with a spigot to provide discharge at the bottom. Further processing was completed on a Tri-Homo disperser[3] serving as an example of a colloid mill, a Manton-Gaulin two-stage laboratory homogenizer,[4] and an Eppenbach Homo mixer[5] typifying the action of a high shear mixer.

The equipment used, the operating conditions employed, and the order of processing are given in Fig. 1. The 20-L. batch of each suspension was agitated by the wall mixer, with a 50-ml. sample being withdrawn for later rheological evaluation after a total elapsed time of five and fifteen minutes of mixing. At the end of the fifteen-minute mixing period, eighteen 500-ml. portions were withdrawn, one at a time, and processed by each of the types of dispersion equipment at the settings indicated in Fig. 1. The processing conditions given in Fig. 1 refer to the condition that was varied during the study. In addition, the homogenizer was operated with the second stage pressure maintained at 500 p.s.i. Pressure was read directly from the gauge supplied with the apparatus. The colloid mill was operated with a rotor-stator clearance of 0.001 inch. The high shear mixer was equipped with a 5.5 cm. impeller. A cylindrical stainless steel container 12.5 cm. in diameter was used for high shear mixing. The mixer shaft was entered vertically nearly to the bottom of the container, a stop being present on the mixer support stand to insure exact reproduction of shaft depth entrance. In operation, the deflector plate of the mixer was positioned just below the level of the liquid and adjusted so that minimum splashing occurred. Operating speeds for the high shear mixer and colloid mill were obtained and regulated by means of a Strobotac.[6] In view of the fact that the dispersion equipment used in the processing of suspensions is generally operated as a line operation, each suspension sample was passed only once through each piece of dispersion equipment, except in the case of the high shear mixer where a mixing time of two minutes was established. The operating conditions employed with each piece of dispersion equipment were chosen to reflect as nearly as possible the conditions encountered in actual practice. Removal of the samples for further rheological processing was accomplished while the processing equipment was at maximum speed or constant pressure.

Storage of Suspensions.—Prior to rheological evaluation, the processed suspensions were stored in tightly closed, one-ounce amber containers for a minimum of sixty days to allow system equilibrium to be established.

Rheological Evaluation.—After storage, flow curves were obtained for each suspension sample by means of a completely instrumented Ferranti-Shirley cone plate viscometer (7, 8, 9). The flow curves were plotted automatically on an X-Y recorder[7] with a full cycle time of sixty seconds and a maximum shear rate of approximately 650 r.p.m.

[1] Johns Manville, New York, N. Y.
[2] R. T. Vanderbilt Co., New York, N. Y.
[3] Tri-Homo Corp., Salem, Mass.
[4] Manton-Gaulin Manufacturing Co., Inc., Everett, Mass.
[5] Gifford-Wood Co., New York, N. Y.
[6] General Radio Corp., Chicago, Ill.

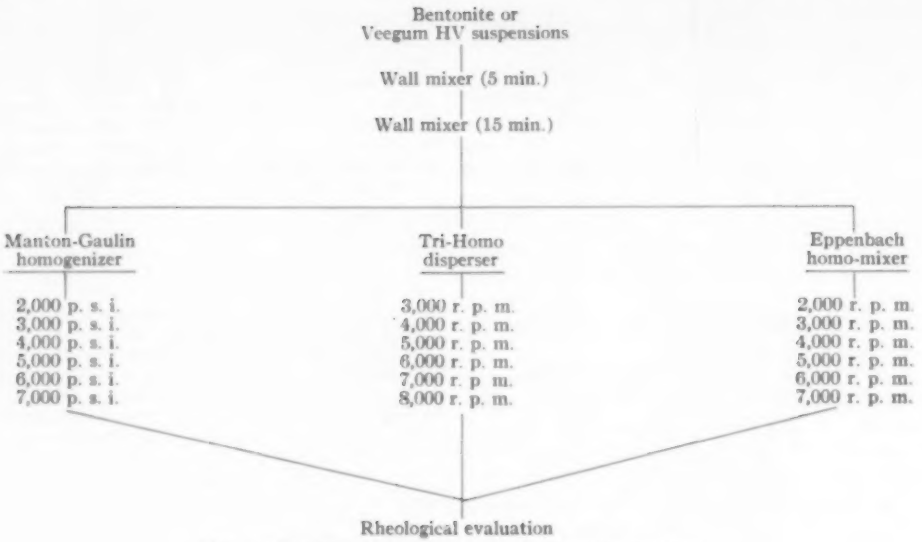

Fig. 1.—Equipment used and order of processing employed.

Temperature was maintained at 25 ± 0.1° while all flow curves were obtained. Temperature control was maintained by means of a Sargent constant temperature bath[8] and the temperature control determined at the surface of the viscometer plate by means of a galvanometer-thermocouple system as described by Hamlow (10). In all procedures care was exercised to perform as little mechanical work on the sample as possible. The nature of the cone plate viscometer is such that little mechanical shear is experienced by the sample as it is placed on the instrument. Investigational procedures were carried out however to satisfy the authors that equilibrium with regard to mechanical breakdown was established within the five to ten minutes the sample was allowed to remain on the plate to reach temperature equilibrium. The viscometer was not operated until the galvanometer-thermocouple system indicated that the suspension sample was at temperature equilibrium with the viscometer plate.

RESULTS AND DISCUSSION

In this study we were interested in evaluating the processed suspension samples in terms of four rheological parameters, thixotropic index, yield value, spur value, and plastic viscosity.

Thixotropic Index.—The thixotropic index is taken as the area circumscribed by the hysteresis loop of the rheogram. The extent of this area depends on several factors, the most significant of which is the maximum speed at which the cone rotates. Thus the values obtained for thixotropic index are relative in nature and in this study expressed as cm.[2]. The areas of the hysteresis loops were determined with the aid of a compensating polar planimeter.[9] The thixotropic indexes thus obtained for each agent and concentration were plotted against the operating variables for each piece of dispersion equipment. Figures 2 and 3 are examples of the results obtained for bentonite 5% suspension and Veegum HV 4% suspension, respectively. Results of the other concentrations are not presented but compare favorably in general form to the examples shown. In addition, Fig. 4 is presented to demonstrate that when the results for several concentrations of the same suspending agent are plotted against the operating variable for one piece of dispersion equipment, the resultant lines are parallel. This indicates that with the concentrations employed, the dispersion equipment tends to cause the same relative change in the thixotropic index. The lines depicted in the graphs were obtained by the method of least squares. Evaluation of thixotropic index indicates a general increase in the parameter as the pressure on the homogenizer is increased. Altering the speed of the colloid mill or high shear mixer appears to have no significant effect on the thixotropic index. Veegum HV suspensions processed on the high shear mixer did appear, however, to exhibit a decrease in thixotropic index as the operating speed was increased Thixotropic indexes for suspensions processed by the colloid mill and high shear mixer were 30 to 50% smaller, depending on operating setting, than for corresponding suspensions processed on the homogenizer.

Yield Value.—The yield value is that shearing stress which must be applied to a thixotropic system before actual flow occurs. Approximate yield values were obtained by extrapolating the linear portion of the downcurve of the rheogram to the X-axis and substituting this value into the appropriate equation (7, 9). A log plot of yield value *vs.* operating variable is presented in Fig. 5 for bentonite 5% suspension. A similar result was obtained for a suspension of bentonite 6%. No yield values were evident for bentonite 4% suspensions. The

[7] F. L. Mosley Co., Pasadena, Calif.
[8] E. H. Sargent Co., Chicago, Ill.
[9] Keufel and Esser Co., Germany.

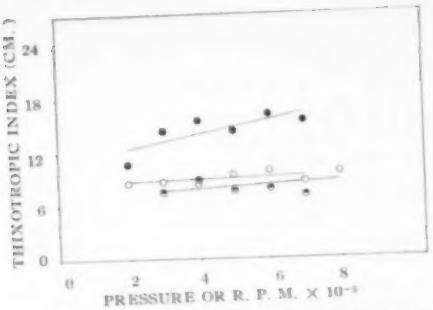

Fig. 2.—Thixotropic index of bentonite 5% suspensions as affected by processing under different conditions by the various types of dispersion equipment. ●, Homogenizer; ◓, colloid mill; ○, high shear mixer.

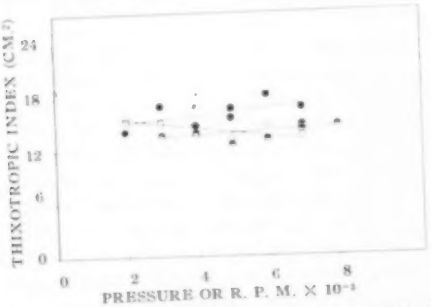

Fig. 3.—Thixotropic index of Veegum HV 4% suspensions as affected by processing under different conditions by the various types of dispersion equipment. ●, Homogenizer; ◓, colloid mill; ○, high shear mixer.

effect of processing on the yield value of Veegum HV suspensions was apparently even greater than that on bentonite suspensions. The variability of the yield value obtained with Veegum HV was however greater than that obtained with bentonite. It is felt that this was due to the greater extent of extrapolation required with the Veegum rheograms, i.e., the apparent plastic viscosity for the Veegum HV suspensions was considerably greater than for the bentonite suspensions and hence the total flow curve made less of an angle with the X-axis in the case of the Veegum HV suspensions. A slight deviation in the angle of the extrapolated line would produce a difference in yield value greater than would be experienced for the bentonite flow curves having the shorter extrapolated line. The tendency in the case of bentonite 5 and 6% suspensions was for the log yield value to increase in a linear manner as the log of the operating variable was increased. Processing by the homogenizer produced suspensions having yield values significantly greater than suspensions processed on the colloid mill and high shear mixer. Differences between suspensions processed on the colloid mill and high shear mixer were not as pronounced but the values for suspensions processed on the colloid mill tended to be greater than for those processed on the high shear mixer.

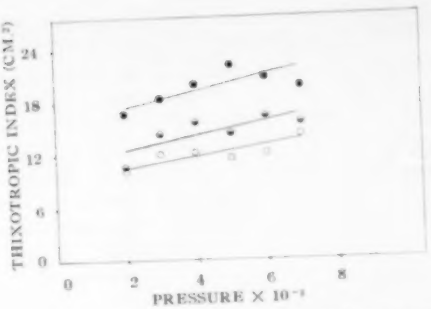

Fig. 4.—Thixotropic index *vs.* operating pressure for bentonite suspensions processed by the homogenizer. ●, 6%; ◓, 5%; ○, 4%.

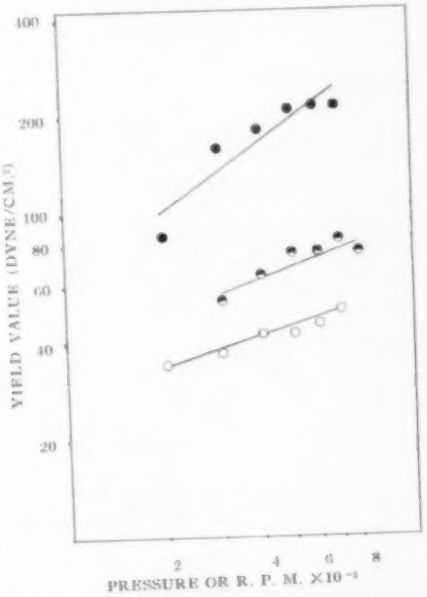

Fig. 5.—Log yield value *vs.* log operating variable for bentonite 5% suspensions processed by the various types of dispersion equipment. ●, Homogenizer; ◓, colloid mill; ○, high shear mixer.

Spur Value.—The spur value is the maximum shearing stress exhibited on the upcurve of the rheogram and is obtained using the same equations that were used for yield value. Because of the conditions of operation of the cone plate viscometer and the X-Y recorder, the spur values were perceived too fleetingly to permit numerical estimates of the magnitudes of these parameters. The fact that the viscometer was automatically cycled and the rheograms were plotted automatically did permit us to make arbitrary estimates of the magnitudes of the spur values and these are presented in Table I. These results indicate that an increase in spur value occurred as the concentration of suspending agent was increased. Spur values were

TABLE I.—MAGNITUDE OF SPUR VALUES OBTAINED WITH VARIOUS CONCENTRATIONS AND TYPES OF DISPERSION EQUIPMENT

	Veegum HV 4%	Veegum HV 5%	Bentonite 4%	Bentonite 5%	Bentonite 6%
Wall mixer	None	Large	None	None	Very slight
Homogenizer	Large	Very large	None	None, slight at higher pressures	Moderate
Colloid mill	Very slight, almost none	Large	None	None	Very slight
High shear mixer	Very slight, almost none	Large	None	None	Very slight

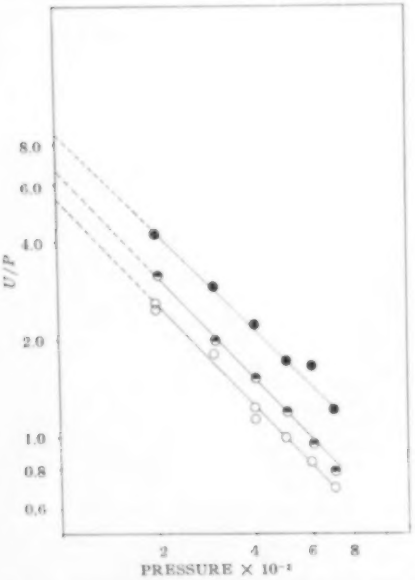

Fig. 6.—Log-log plot of U/P vs. P for suspensions processed by the homogenizer. ●, Bentonite 5%; ◓, Veegum HV 5%; ○, Veegum HV 4%.

strongly evident in all suspensions of Veegum HV but almost entirely absent in the bentonite suspensions. The homogenizer was the piece of dispersion equipment most effective in enhancing this parameter by processing.

Plastic Viscosity.—The plastic viscosity was taken as the reciprocal of the slope of the linear portion of the downcurve of the rheogram. In actuality the plastic viscosity should be taken as the reciprocal of the slope of the tangent to the curve at any one point. The values obtained in this study, however, are significant from a practical point of view in that they estimate the "thickness" of the suspension system after shear has occurred. Investigation of the results and several preliminary plots indicated that the relationship between plastic viscosity and operating variable might be exponential in nature and of the form

$$U/P = \alpha P^{1/\beta}$$

where U is the plastic viscosity in centipoises, P is the operating variable in p.s.i. or r.p.m., and α and β are constants. If such a relationship were true, the results plotted on log-log paper as U/P vs. P should be a straight line with slope $1/\beta$ and Y-intercept log α. The results of such a plot are shown in Fig. 6. The plots appear to have good linear correlation and the fact that the β-values are similar indicates that β might be a function of the type of equipment used. The magnitude of α would necessarily depend on the suspending agent employed and the concentration used. Further comment on the validity of this relationship must be postponed until additional data are obtained.

SUMMARY AND CONCLUSIONS

Suspensions of Veegum HV and bentonite U. S. P. were prepared in several concentrations and processed on three types of dispersion equipment. Flow curves for samples of the suspensions processed under different operating conditions on the dispersion equipment were obtained and the rheological parameters of thixotropic index, yield value, spur value, and plastic viscosity were obtained from these flow curves. The parameters so obtained were evaluated in relation to the type of equipment used for processing, to the suspending agent and concentration involved, and to the operating setting of the processing equipment. The results obtained from the above study were:

1. The rheological parameters which were determined increased with increasing concentration of suspending agent.

2. The rheological parameters of the suspensions processed with the homogenizer were generally greater than for suspensions processed with the colloid mill or high shear mixer.

3. Increasing the operating pressure of the homogenizer resulted in an increase in the thixotropic index of both the bentonite and Veegum HV suspensions. Altering the operating speed of the colloid mill and high shear mixer had little effect on the thixotropic indexes of the bentonite and Veegum HV suspensions, an exception being the Veegum HV suspensions processed by the high shear mixer where the thixotropic index decreased as the operating speed was increased.

4. The log of the yield value appeared to be a linear function of the log of the operating variable of the processing equipment for the bentonite 5 and 6 per cent suspensions.

5. The relationship between the plastic viscosity and processing operating variable for the

suspending agents studied in several concentrations appeared to follow the equation

$$U/P = \alpha P^{1/\beta}$$

6. The magnitude of the spur value was much greater for the Veegum HV suspensions than for the bentonite suspensions. Processing by the homogenizer tended to increase the spur value to a greater extent than any of the other types of dispersion equipment employed.

The results indicate that as the suspensions were subjected to increasing shearing rates or forces, the rheological parameters increased in magnitude. It is not surprising to find, in addition, that the rheological parameters are affected to their greatest extent by the homogenizer, as it is the apparatus designed to subject the suspension particles to the greatest amount of shear. We can attribute the increase in magnitude of the rheological properties to the increased hydration brought about by the shearing forces dispersing the flocculated particles of the system. The suspensions studied were processed under conditions selected to include operating settings in their upper limits and, since maxima of rheological properties were not noted, it is doubtful if complete hydration is ever achieved under normal operating conditions. It is therefore necessary to include in the formulation of a suspension an excess of suspending agent above that which would have been required if complete hydration could be achieved. However, we can increase the extent of hydration and hence the rheological parameters by mechanical action. The effect of processing on the parameters then has economic significance with regard to the use of both equipment and material. In addition, a consideration of the effects of processing on the rheological parameters of thixotropic suspensions may lead to a better insight into the formulation and production of liquid suspensions.

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Notes

Nonaqueous Titration of Chlorothiazide-Type Diuretics

By H. C. CHIANG

Chlorothiazide-type diuretics in dimethylformamide were titrated with sodium methoxide using *p*-nitrobenzene-azo-resorcinol as indicator. The method is proposed for the rapid analysis of pure crystalline compounds and pharmaceutical products.

THE WIDE USE and the therapeutic importance of saluretic agents have resulted in an increased number of chlorothiazide-type diuretics. Chlorothiazide, dihydrochlorothiazide, flumethiazide, dihydroflumethiazide, and bendroflumethiazide are new oral nonmercurical saluretics and diuretics. In spite of recent emphasis upon these derivatives, very few analytical methods were published. Apparently little or no work has been reported on the method of determining flumethiazide, dihydroflumethiazide, and bendroflumethiazide. The literature presents only the spectrophotometric method for chlorothiazide (1–3) and dihydrochlorothiazide (4), and nonaqueous titration of chlorothiazide (5). Recently, perchloric acid and lithium methylate were used for the nonaqueous titration of these compounds (6). In the U. S. P. XVI procedure (5), chlorothiazide in dimethylformamide is titrated with sodium methoxide, using thymol blue as indicator. But the method does not show a sharp color change at the end point. For this reason, a more suitable condition for nonaqueous titration was sought. As a result of this study, a rapid and convenient method has been developed for the determination of all these type diuretics.

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Reagents.—Dimethylformamide; benzene; methanol absolute; benzoic acid; *p*-nitrobenzene-azo-resorcinol indicator: saturated benzene solution of p-nitrobenzene-azo-resorcinol; and 0.1 *N* sodium methoxide (U. S. P., except standardization is with three drops of *p*-nitrobenzene-azo-resorcinol indicator, titrated with sodium methoxide to a blue end point).

Procedure.—*Pure crystalline.*—Dissolve about 250 mg. of sample (accurately weighed) in 80 ml. of dimethylformamide in a flask by stirring electromagnetically and observing precautions against absorption of atmospheric carbon dioxide. Then add three drops of *p*-nitrobenzene-azo-resorcinol indicator and titrate with 0.1 *N* sodium methoxide to a blue color as the end point. Perform a blank determination, and make any necessary correction. Each ml. of 0.1 N sodium methoxide is equivalent to 14.787 mg. of chlorothiazide ($C_7H_6ClN_3O_4S_2$), 14.888 mg. of dihydrochlorothiazide ($C_7H_8ClN_3O_4S_2$), 16.465 mg. of flumethiazide ($C_8H_6F_3N_3O_4S_2$), 16.565 mg. of dihydroflumethiazide ($C_8H_8F_3N_3O_4S_2$), and 21.072 mg. of bendroflumethiazide ($C_{15}H_{14}F_3N_3O_4S_2$).

Commerical Preparations.—Weigh and powder twenty tablets. Place an accurately weighed sample equivalent to 250 mg. of diuretic in a flask, then proceed as above.

RESULTS AND DISCUSSION

Vespe and Fritz (7) showed that sulfa drugs in dimethylformamide titrated with sodium methoxide using thymol blue as indicator gave sharp visual end points. This procedure is used in U. S. P. XVI for the determination of chlorothiazide (5). The author has tried the method with all these type diuretics and found it to give unsatisfactory end points. Chlorothiazide-type diuretics titrated using *p*-nitrobenzene-azo-resorcinol as indicator give sharp end points. At the end point, each mole of diuretics is equivalent to two moles of sodium methoxide. For chlorothiazide and flumethiazide, two end points were obtained. At the first end point, the color changed from yellow to pink and at the second end point, it changed from pink to blue. In this case, each mole of diuretics is equivalent to one mole of sodium methoxide at the first end point and two moles of sodium methoxide at the second end point. For best results, the solution should be protected from carbon dioxide in atmosphere during the titration. The proposed method of assay was applied to the pure crystalline and pharmaceutical products. The percentage of recovery at these end points are shown in Table I. Also, a comparison was made between the results obtained by the spectrophotometric method for chlorothiazide and dihydrochlorothiazide

TABLE I.—ANALYSIS OF PURE CRYSTALLINE DRUG AND PREPARATIONS

Drug	Recovered, %	
Method →	Nonaqueous[a]	Spectrophotometry
Chlorothiazide U. S. P.	99.12 (99.23)	99.08
Chlorothiazide, 250 mg. tab.	101.78 (101.51)	101.92
Flumethiazide	98.87 (98.45)	...
Flumethiazide, 250 mg. tab.	104.14 (103.76)	...
Dihydrochlorothiazide	98.83	98.87
Dihydrochlorothiazide tab., 50 mg./tab.	103.11	102.79
Dihydrochlorothiazide with resperine tab.; dihydrochlorothiazide, 25 mg., reserpine, 0.125 mg./tab.	104.59	107.44
Dihydroflumethiazide	99.23	...
Dihydroflumethiazide, 50 mg. tab.	103.61	...
Bendroflumethiazide	98.87	...

[a] Numbers within parentheses represent recovery at the first end points.

From the results shown in Table I one can clearly note close agreement in recoveries determined by two methods. For dihydrochlorothiazide with reserpine tablets, the spectrophotometric method gave a higher result because of the ultraviolet absorption of reserpine. Most common tablet excipients such as potato starch, magnesium stearate, and kaolin give negligible blanks. Reserpine and potassium chloride, which frequently occur with these diuretics in pharmaceuticals, do not interfere. However, 0.5 Gm. of sugar and lactose required approximately 4 ml. of 0.1 *N* sodium methoxide.

SUMMARY

The nonaqueous method is applied to a variety of chlorothiazide-type diuretics. The procedure is particularly suitable for pure crystalline and tablets. Sugar and lactose interfere with the method.

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Preparation of Crystalline Pilocarpine Methiodide

By NORBERT J. WOJCIECHOWSKI and BERNARD ECANOW

A method for preparing crystalline pilocarpine methiodide is reported. Pilocarpine hydrochloride is converted to isopilocarpine hydrochloride in good yields by a convenient method. A comparison of the melting points, mixed melting points, and optical rotation data of pilocarpine methiodide and isopilocarpine methiodide show them to be different substances and not interconverted in the synthetic procedure developed.

THE PREPARATION of pilocarpine methiodide has been reported by a number of workers. The salt has always been reported in the literature as an oil which could not be crystallized (1–3).

The stereoisomer of pilocarpine, isopilocarpine, is reported in the literature as the crystalline methiodide salt with a melting point of 114°.

A method for preparing the crystalline pilocarpine methiodide is reported in this paper. The preparation of pilocarpine methiodide required five weeks in the first synthesis, during which period it might have converted to the stereoisomer. The stereoisomer, isopilocarpine methiodide, was prepared and the two methiodide derivatives compared.

A convenient method for converting pilocarpine hydrochloride to isopilocarpine hydrochloride in good yield is also reported.

EXPERIMENTAL

Preparation of β-(Hydroxymethyl)-α-ethyl-1,3-dimethyl Imidazolebutyric Acid Lactone Iodide (Methyl Pilocarpinium Iodide or Pilocarpine Methiodide).—In order to obtain pilocarpine base for the alkylation, a sample of pilocarpine hydrochloride U. S. P., Mallinckrodt, weighing 6.99 Gm. was dissolved in distilled water, the solution made alkaline, and extracted with portions of chloroform until the aqueous solution gave a negative Helch test. The combined chloroform solutions were washed with distilled water, filtered, and concentrated by evaporation until a limp oil remained. Methyl iodide (5 ml.) was added to the flask and the stoppered flask allowed to stand twelve hours. Twenty five milliliters of absolute alcohol was added to the flask and enough ether was then added, with swirling, until the solution became permanently turbid. The stoppered flask was then placed in a refrigerator for five hours. The supernatant liquid was then decanted from a precipitated yellow oil. The yellow oil was dissolved in alcohol and reprecipitated with the addition of ether and cooling in a refrigerator. This procedure was repeated for a total of five times. The yellowish oil was then collected in a glass vial and the vial placed in warm water to drive off the residual ether. The capped vial was stored in a vacuum desiccator over Drierite and protected from light.

A re-examination of the product after five weeks showed that the oil had changed to a solid yellowish mass resembling yellow wax. The thin material on the sides of the vial appeared crystalline. Scraping the solid with a spatula yielded a yellowish-white granular material with a melting point of 121–123°. Some of the solid was saved to be used as seed crystals.

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The remainder of the solidified mass was dissolved in alcohol, the solution decolorized with Norit A, and filtered. Ether was added until the point of first turbidity and the solution placed in the freezing compartment of a refrigerator. Within an hour there was precipitation of a light yellow oil which settled to the bottom of the flask. A few seed crystals were added to the solution and crystal growth was initiated. When the crystals fell into the collected oil it also became crystalline. After crystallization was complete (no further turbidity upon the addition of more ether) the crystals were collected on a Büchner funnel. These crystals had a melting point of 122–124°.

When the synthesis was repeated, it was found that the addition of a few seed crystals to the yellow oil rapidly induced crystallization without numerous purifications or prolonged storage of these batches. Yields of 87.4 to 90.0% were obtained.

The material collected from the first synthesis was combined with the crystalline solid obtained from a second synthesis. The pooled sample was dissolved in absolute methanol and the solution treated with Norit A. The solution was filtered into an Erlenmeyer flask and anhydrous ether was added to the alcohol solution in small portions almost to the point of permanent turbidity and with no precipitation of oil droplets. A few seed crystals were added to the flask and the clear solution was placed in a refrigerator. In twelve hours the precipitated white cubical crystals were collected on a Büchner funnel by means of vacuum filtration (water aspirator). The crystals were dried in a desiccator. The crystals had a melting point (Hoover-Thomas capillary melting point apparatus) of 122.5–125°. These crystals on microanalysis showed values of N, 8.08; I, 36.08%. The calculated values for methyl pilocarpinium iodide ($C_{11}H_{16}N_2O_2 \cdot CH_3I$) are N, 8.88; I, 36.24%.

Conversion of Pilocarpine Hydrochloride to Isopilocarpine Hydrochloride.—Ten grams of pilocarpine hydrochloride was weighed into a 100-ml. beaker and placed uncovered in a 200° oven. After two hours, the melted material was removed from the oven and allowed to cool. When cool, it formed a transparent, blackish-brown glass. The mass was dissolved in 25 ml. of warm absolute alcohol. The resulting yellow solution was decolorized with the use of Norit A and filtered. Ether was added to the cooled alcohol solution until a slight turbidity persisted. The beaker was covered and placed in the freezing compartment of a refrigerator. In twelve hours the white crystals that had formed were collected, redissolved in alcohol, and filtered. Ether was added to the filtrate until a permanent turbidity persisted. The flask was placed back in the refrigerator. In twelve hours the salt was collected on a Büchner funnel and the filtrate discarded.

TABLE I.—SPECIFIC ROTATIONS

Compound	$[\alpha]_D^{25}$	Concn., Gm.	Reported Literature Value, °C.
Pilocarpinium methyl iodide	+62.08	4.027	New
Isopilocarpinium methyl iodide	+25.26	3.998	26 (18)

The crystals were washed with several small portions of ether and then placed in a desiccator overnight. The melting point of the crystals was 120–123°. The melting point of pure, air-dried isopilocarpine hydrochloride as reported by Jowett (3) is 127°. He also reports that isopilocarpine hydrochloride contains a one-half mole of water of crystallization, and when dried to constant weight at 110° the melting point of the anhydrous salt is 159°.

The isopilocarpine hydrochloride obtained was placed in a 110° oven and dried for three and one-half hours. The melting range of the dried salt was 155–158°. The material was used for the preparation of the methiodide.

Preparation of Methyl Isopilocarpinium Iodide.—The procedure followed was essentially that used to prepare the methyl pilocarpinium iodide. Recrystallization from alcohol gave white needle crystals with a melting point of 112–114° (reported melting point 114°).

Optical Rotations.—The specific rotations of the two compounds were determined by measurement in a polarimeter with water as the solvent at 20°. The results are shown in Table I.

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Exhaustive Methylation of Glucosamine

By NORBERT J. WOJCIECHOWSKI, RALPH DANIELS, and BERNARD ECANOW

A reported synthesis of a glucosamine quaternary derivative was investigated by chemical and infrared spectra methods. Under the conditions reported the glucosamine was found to become degraded and tetramethylammonium iodide was formed.

AN EXCELLENT REVIEW of the chemistry of the glycosylamines appears in a publication by Ellis and Honeyman (1). Additional articles may be found in Pigman's text (2) and in the publication by Kent and Whitehouse (3).

An interest in the preparations of unsubstituted -O- quaternary derivatives of glucosamine prompted an investigation into the procedure reported by Coles and Bergeim (4).

EXPERIMENTAL

The procedure followed for the synthesis of a glucosamine quaternary derivative was that reported by Coles and Bergeim (5). The synthesis was repeated five times to enable the close observation of experimental conditions and reactions.

Analysis and Summary of Experimental Procedure.—The action of methyl iodide and potassium hydroxide upon glucosamine under the conditions reported results in the formation of a compound with a high decomposition point. Under the conditions of the reaction, potassium iodide is one of the products formed. It is coprecipitated with the product and is therefore a contaminant which is removed only after much processing. The explanation proposed by Coles and Bergeim for the increased percentage of iodine over the calculated theoretical value is unsatisfactory on the basis of additional microanalytical work.

Anal.—Calcd. for $C_9H_{20}INO_5$: C, 30.96; H, 5.77; I, 36.35; N, 4.01; O, 22.91. Found (4, 5): C, 23.89, 24.03; H, 5.62, 6.09; I, 62.90, 62.90; N, 7.16, 6.65.

The iodine values compare with those reported by these authors, 61.71% (Paar bomb), 61.39% (gravimetric), but are well in excess of the theoretical values for a glucosamine derivative. Inspection of the microanalytical results show the absence of any oxygen and therefore does not support the existence of a glucosamine derivative which would contain 22.91% oxygen. An average of the analytical results obtained indicates an atomic ratio of 1 nitrogen, 4 carbon, and 12 hydrogen atoms for each iodine atom, or 1 iodine, 4 carbon and 12 hydrogen atoms for each nitrogen atom, giving an empirical formula of $C_4H_{12}IN$. The iodine appears to be ionic; the compound is soluble in water and insoluble in ether. This would satisfy the general characteristics for a quaternary salt. A quaternary salt having the empirical formula $C_4H_{12}IN$ could be represented by tetramethylammonium iodide, $(CH_3)_4NI$. Tetramethylammonium iodide has a reported density of 1.84. A few crystals placed in carbon tetrachloride, density 1.59, sink to the bottom of the test tube but float on the surface of ethyl iodide, density 1.95.

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The decomposition point of a sample of tetramethylammonium iodide was determined by a capillary tube in a silicone bath. The salt shrank in the capillary at about 370°. It was blackened and almost consumed by about 380°. A mixed decomposition point with some of the isolated crystals was determined. It had the same general characteristics as the pure salt, i. e., browning at about 370° and blackened by about 385°. An infrared analysis was performed on a sample of tetramethylammonium iodide and on the isolated product using a Beckman infrared spectrophotometer model IR-4. The infrared spectrum of tetramethylammonium iodide is identical to that obtained for tetramethylammonium bromide (6). The infrared spectrum of the "glucosamine derivative" shows no absorption in the alcoholic hydroxyl band (3,300 $cm.^{-1}$) (7), indicating the absence of carbohydrate. When superimposed on one another, the spectra of the "glucosamine derivative" and tetramethylammonium iodide are identical. This is satisfactory proof that the product of the alkylation of glucosamine under the experimental conditions leads to the production of tetramethylammonium iodide.

The ether extracts that were obtained when the pasty brown crystalline mass was washed were combined and analyzed in an attempt to isolate other degradation products of glucosamine. The extracts were decolorized as much as possible with Norit and filtered into a beaker. The ether was evaporated to dryness. The residue which remained after evaporation was dissolved in a few milliliters of distilled water. The solution was divided into three portions. A dark green color was produced upon the addition of two drops of ferric chloride T. S. to a portion of the solution. A second portion added to a silver nitrate solution resulted in immediate reduction of the silver nitrate. Fehling's solution was reduced when heated to boiling with the last portion of solution. The above tests are suggestive of the presence of pyrocatechol, shown by Ledderhose (8) to be a degradation product of glucosamine hydrochloride in alkaline solution.

The suggestion by Coles and Bergeim that the glucosamine derivative has a pharmacologic interest in view of its choline-like action prompted a further investigation. It is proposed in this paper that the product obtained is devoid of glucosamine structure and is the quaternary salt, tetramethylammonium iodide. That tetramethylammonium iodide is a physiologically active substance may be found in numerous journal articles and textbooks of pharmacology. That the action of the "glucosamine derivative" is satisfied by the pharmacology of tetramethylammonium iodide is apparent in these articles. The muscarine-like and nicotine-like circulatory activities of methyl and ethyltrimethylammonium compounds are reported in articles by Burn and Dale (9) as early as 1915, and by Hunt (10) in 1926. In a paper by Alles and Knoefel, a comparative study was made on the muscarine-like and nicotine-like actions of the whole series of methyl- to nonyl-trimethylammonium iodides. The investigation included different animals and, in particular, was directed to the action on the circulatory system, intestine, and muscle. The muscarinic and nicotinic effects vary markedly and pass through maxima in the series methyl to nonyl. The effects on arterial pressure following intravenous injections were observed. Observations were made before and after atropine in dosage adequate to abolish the depressor effects of these salts in order to measure independently their muscarine-like and nicotine-like activities. With dosage of the alkyltrimethylammonium salts in the range of 10^{-7} to 10^{-6} moles per Kg. (0.02 to 0.2 mg./Kg.), both depressor and pressor effects were observed leading to a diphasic response, the depressor effect being abolished by pretreatment with atropine. The nicotine-like activities of the more active of these salts are greater than that of acetylcholine, whereas the muscarine-like activities are about one-fiftieth to one-hundredth that of acetylcholine. The species response is also pointed up where the nicotinic effects in the rabbit are much less than in the dog. There is much less species difference in muscarine-like depressor action (11).

It would appear that the response to alkyl-trimethylammonium salts, and specifically in regard to tetramethylammonium iodide, is the same pharmacological action that is reported for the "glucosamine derivative."

SUMMARY

1. The product obtained by the alkylation of glucosamine hydrochloride in hot alkaline solution is not quaternized glucosamine but tetramethylammonium iodide.

2. The identification of pyrocatechol as one of the products gives further evidence for the degradation of glucosamine.

3. The infrared spectrum of the isolated product shows the absence of any carbohydrate and confirms the existence of tetramethylammonium iodide.

4. The pharmacologic activity reported for alkyl-trimethylammonium iodide is compared with the reported pharmacologic activity of the "glucosamine derivative" and found to be identical.

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Control of Urine pH and Its Effect on Drug Excretion in Humans

Sir:

We wish to report a technique which makes it possible to have urine excreted at constant pH during drug excretion studies in humans. Using this procedure it was possible to study the rate of urinary excretion of sulfaethidole over periods of approximately ten to twenty hours with urinary pH maintained constant to ±0.1 pH in the vicinity of pH 5 or pH 8. It is more difficult to maintain constant pH at intermediate pH values

In the pH range 4.5 to 5.0, pH is easily maintained constant to ±0.1 pH by administering an initial dosage of approximately 3 to 4 Gm. of ammonium chloride every hour for two or three hours until the desired pH has been attained, and then administering smaller doses as indicated by hourly monitoring of urine pH. Similar constancy can be obtained at pH of approximately 8.0 by administering initial hourly doses of approximately 4 Gm. of sodium bicarbonate, followed by smaller hourly doses after the desired pH has been attained. Total intake of ammonium chloride during the course of an excretion study ranged from 10 to 15 Gm., and total intake of sodium bicarbonate during a study ranged from 30 to 48 Gm. For most trials, food and fluid intake were not restricted.

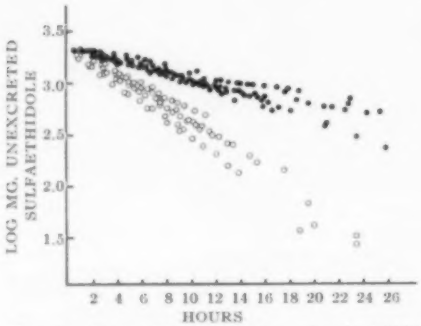

Fig. 1.—First-order elimination of sulfaethidole after a 2-Gm. oral dose in humans. ●, urine maintained at pH 5; ○, urine maintained at pH 8.

Figure 1 is a composite plot of 31 separate excretion studies on 12 individuals. The significance of controlled urinary pH in excretion studies on drugs such as sulfaethidole is clearly indicated. A complete report of this work will be published.

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ERRATUM

In the paper titled "Pharmacological Studies on Some New Azlactone Derivatives" (1), Compound No. 10, R^1 should read 3-pyridyl instead of 4-pyridyl.

(1) Robison, G. A., and Schueler, F. W., THIS JOURNAL, **50**, 602(1961).

ERRATUM

In the paper titled "Qualitative and Quantitative Tests for Isobucaine Hydrochloride" (1), the infrared spectrum given was not that of isobucaine hydrochloride. The correct spectrum is shown in Fig. 1.

(1) Feldmann, E. G., Hefferren, J. J., Koehler, H. M., and Reasenberg, J. R., THIS JOURNAL, **50**, 347(1961).

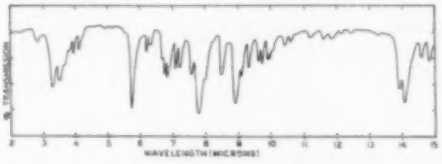

Figure 1.

Contents continued from back cover

NORBERT J. WOJCIECHOWSKI, BERNARD ECANOW — 854 — Synthesis of Some Quaternary Pilocarpine Salts

L. J. LEESON, J. A. LOWERY, G. M. SIEGER, C. KRIEGER — 856 — Steroid Borates III. Use in Preparation of Triamcinolone 21-Hemisuccinate and Other 21-Monoesters

ELBERT VOSS, A. R. HASKELL, L. GARTENBERG — 858 — Reduction of Tetramine Toxicity by Sedatives and Anticonvulsants

A. P. SIMONELLI, T. HIGUCHI — 861 — Dilatometric Determination of Relatively Pure Systems

RONALD E. ORTH, JAMES W. JONES — 866 — Cyclized Substituted Thioureas I. Preparation of Some 3-Substituted 1,2,3,4-Tetrahydroquinazoline-2-thiones and Their Intermediates

G. M. SIEGER, W. C. BARRINGER, F. M. CALLAHAN, N. GRUENFELD, J. P. WEIDENHEIMER — 869 — Mannich Bases of Phenolsulfonamides: Derivatives of N-(2-Thiazolyl)-1-phenol-4-sulfonamide

TAKERU HIGUCHI — 874 — Rate of Release of Medicaments from Ointment Bases Containing Drugs in Suspension

N. S. DHALLA, M. S. SASTRY, C. L. MALHOTRA — 876 — Chemical Studies of the Leaves of *Withania somnifera*

STEVEN G. BJAASTAD, NATHAN A. HALL — 878 — Effects of Ethylenediaminetetraacetic Acid (EDTA) on Prothrombin Consumption

Technical Articles

THOMAS H. SIMON, H. GEORGE DEKAY, GILBERT S. BANKER — 880 — Effects of Processing on the Rheology of Thixotropic Suspensions

Notes

H. C. CHIANG — 885 — Nonaqueous Titration of Chlorothiazide-Type Diuretics

NORBERT J. WOJCIECHOWSKI, BERNARD ECANOW — 887 — Preparation of Crystalline Pilocarpine Methiodide

NORBERT J. WOJCIECHOWSKI, RALPH DANIELS, BERNARD ECANOW — 888 — Exhaustive Methylation of Glucosamine

Communications

JOEL D. PORTNOFF, JOSEPH V. SWINTOSKY, H. B. KOSTENBAUDER — 890 — Control of Urine pH and Its Effect on Drug Excretion in Humans

890 — Errata

JOURNAL OF **Pharmaceutical Sciences**

October 1961 volume 50, number 10

Editorial

I Walter H. Hartung, Ph.D.

Review Article

WALTER H. HARTUNG, JOHN ANDRAKO — 805 Stereochemistry

Research Articles

S. MORRIS KUPCHAN, WADY L. ASBUN, B. S. THYAGARAJAN — 819 Menispermaceae Alkaloids III. Alkaloids of *Stephania hernandifolia*

M. D. ACETO, V. D. LYNCH, R. K. THOMAS — 823 Effects of Drugs on Conditioning in the Rat II. Synthesis of a Centrally Active Drug and the Effects of Nine Drugs on Operant Conditioning and Extinction

L. J. VINSON, E. L. AMBYE, A. G. BENNETT, W. C. SCHNEIDER, J. J. TRAVERS — 827 *In Vitro* Tests for Measuring Antibacterial Activity of Toilet Soap and Detergent Bars

HENRY LEMAIRE, C. H. SCHRAMM, ARNO CAHN — 831 Synthesis and Germicidal Activity of Halogenated Salicylanilides and Related Compounds

CHARLES MILOS — 837 Spectrophotometric Assay of Opium and Paregoric

P. N. KAUL, E. BROCHMANN-HANSSEN, E. LEONG WAY — 840 Biological Disposition of Apomorphine IV. Isolation and Characterization of "Bound" Apomorphine

WAYNE V. KESSLER, GLENN L. JENKINS — 842 Synthesis of N-Substituted Methanedisulfonamides and N′-Substituted Methanedisulfonylureas

ALFRED F. ZAPPALA, CHARLES A. SIMPSON — 845 Sensitive Colorimetric Method for the Determination of Panthenol in Multivitamin Preparation

E. G. CLAIR, L. G. CHATTEN — 848 Determination of Antihistamines in Pharmaceutical Products by Nonaqueous Titration

Contents continued on inside back cover